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Characterization of M-cadherin's Role in Regulating Apoptosis and Myogenic Differentiation of Myoblasts by Interacting with Key Components of Canonical Wnt Signaling

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Characterization of M-cadherin's Role in Regulating Apoptosis and Myogenic Differentiation of Myoblasts by Interacting with Key Components of Canonical Wnt Signaling

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Dissertation submitted to the School of Medicine

at West Virginia University

In partial fulfillment of the requirements for the degree of

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in

Exercise Physiology

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2011

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ABSTRACT

Characterization of M-cadherin's Role in Regulating Apoptosis and Myogenic Differentiation of Myoblasts by Interacting with Key Components of Canonical Wnt Signaling

Yan Wang

Activation, proliferation, and differentiation of satellite cells are the basic means for adult muscle regeneration. Apoptosis is concurrent to differentiation of satellite cells and dysregulated apoptosis contributes to the occurrence and progression of certain muscular dystrophies. This study investigated the role of M-cadherin-mediated signaling in regulating apoptosis versus survival of myoblasts during the process of myogenic differentiation. Inhibition of M-cadherin expression by RNAi (RNA interference) in confluent C2C12 myoblasts sensitized the cells to mitochondria-associated intrinsic apoptosis induced by either cell confluence or serum starvation. Manipulation of M-cadherin signaling regulated the Glycogen Synthase Kinase-3 β (GSK-3 β) activity via phosphoinositide-3 kinase (PI3K)/Akt pathway. Overexpression of wild-type (WT) GSK-3 β in confluent C2C12 myoblasts sensitized the cells to apoptotic insults, while GSK-3 β inhibition attenuated apoptosis and partially rescued the myogenic differentiation impaired by M-cadherin RNAi in both C2C12 myoblasts and Syndecan-4-positive primary myoblasts. These data suggest that M-cadherin-mediated signaling protects myoblasts against mitochondria-associated intrinsic apoptosis during myogenic differentiation via PI3K/Akt/GSK-3 β pathway. We further examined the role of M-cadherin in regulating the N-terminal phosphorylation status of β -catenin and the effect of this regulation on myoblast fate specification. M-cadherin RNAi enhanced the GSK-3 β -dependent phosphorylation of β -catenin at N-terminus which can be reversed by GSK-3 β inhibition via LiCl treatment. N-terminus unphosphorylated β -catenin was more responsive to LiCl treatment. In addition, M-cadherin RNAi led to an increase in TCF/LEF transcription activity but significantly abrogated the myogenic differentiation induced by LiCl or Wnt-3a treatment. β -catenin RNAi also blocked the myogenic induction by LiCl or Wnt-3a. Although forced expression of a phosphorylation-resistant mutated β -catenin (S33Y- β -catenin) failed to increase myogenic differentiation, it partially rescued the impaired myogenic differentiation and attenuated the apoptosis caused by M-cadherin RNAi. These data indicate that M-cadherin-mediated signaling plays a positive role in maintaining a cytosolic pool of signaling-active N-terminal unphosphorylated β -catenin, which is critical for the TCF/LEF-independent myogenesis-promoting effect of canonical Wnt signaling. Our findings in this research identify a novel role of M-cadherin in regulating myoblasts survival and differentiation and provide a potentially novel molecular mechanism for the regulation of adult muscle regeneration.

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LIST OF ABBREVIATIONS

α	alpha
ABC	unphosphorylated signaling-active β -catenin
Apaf-1	Apoptosis protease activating factor-1;
APC	adenomatous polyposis coli
β	beta
β-TrCP	β -transducin repeat-containing protein
bHLH	basic helix-loop-helix
$^{\circ}\text{C}$	degree Celsius
CD	cluster of differentiation
CKIα:	casein kinase I α
CuSOD	copper-zinc superoxide dismutase
Cyt <i>c</i>	cytochrome <i>c</i>
DAPI	4,6-diamidino-2-phenylindole
DGC	dystroglycan complex
DISC	death-inducing signaling complex
DKK-1	Dickkopf-related protein 1
DMD	Duchenne Muscular Dystrophy
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulfoxide
ECL	enhanced chemiluminescence lighting
ECM	extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EndoG	endonuclease G
ER	endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FADD	Fas-associating death domain-containing protein

FBS	fetal bovine serum
FGF	fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GSK-3β	Glycogen synthase kinase-3 β
GSK-3βK85R	kinase-deficient mutant GSK-3 β
HGF	hepatocyte growth factor
HRP	horseradish peroxidase
HSF-1	Heat Shock Factor-1
IKKα	I κ B kinase α
I-mfa	an Inhibitor of the MyoD family of mouse MRFs
IMS	intermembrane space
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine Iodide
JNK	c-Jun N-terminal kinases
kDa	kiloDalton
LEF	lymphoid enhancer factor
LiCl	lithium chloride
LRP5/6	low density lipoprotein related proteins 5 & 6
MAPK	Mitogen-activated protein kinase
MnSOD	Manganese Superoxide Dismutase
μ	micro
$\Delta\psi_{mt}$	mitochondria membrane potential
MOMP	mitochondrial outer membrane permeabilization
MPCs	muscle progenitor cells
MRFs	myogenic regulatory factors
MSC	mesenchymal stromal cells

MyHC	myosine heavy chain
myrAkt	myristoylated Akt mutant
NAO	Nonyl acridine orange
NFAT	Nuclear factor of activated T-cells
NO	nitric oxide
p90RSK	p90 ribosomal S6 kinase
PARP	Poly ADP ribose polymerase
PKD1	phosphoinositide dependent kinase
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol (3,4,)-biphosphate
PIP3	phosphatidylinositol (3,4,5)-triphosphate
PKA	cyclic AMP-dependent protein kinase
PKB	protein kinase B
RNAi	RNA interference
SC	satellite cells
siRNA	small interfering RNA
Smac/DIABLO	second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with a low isoelectric point
TBS	Tris buffered saline
TCF	T cell factor
TDZD	Thiadiazolidinones
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
WT	wild type

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SPECIFIC AIMS

Differentiation of satellite cells and fusion into myotubes is the basic means for adult muscle regeneration (1; 2). Apoptosis is a concurrent event to myogenic differentiation, that is, a substantial portion of cells die of apoptosis as the majority of cells differentiate and fuse into myotubes (3; 4). The molecular mechanism by which the myoblasts make the life versus the death decision remains unclear. Cadherins are calcium-dependent homophilic cell adhesion molecules forming adherens junctions and desmosomal junctions between cells. M-cadherin is a member of classical cadherins that is specifically expressed in skeletal muscle and certain neural tissues (5; 6). It is reported that M-cadherin mediates the cell-cell fusion and is required for myogenic differentiation (7; 8). However, its role in regulating myoblast survival/apoptosis has never been addressed.

β -catenin/canonical Wnt signaling plays a critical role in myogenesis and adult muscle regeneration (9; 10). β -catenin is an essential mediator for canonical Wnt signaling as well as a key adaptor protein that connects M-cadherin to α -catenin and the actin cytoskeleton system (11). Cadherin has an established role as a β -catenin signaling inhibitor by either mechanical sequestration or transcriptional suppression (12-14). A recent study showed that E-cadherin in colon carcinoma cells could directly suppress β -catenin activity by promoting its phosphorylation at the N-terminus by GSK-3 β (15). Sequential phosphorylation of β -catenin at the N-terminus by CK1 α and GSK-3 β results in its recognition by β -TrCP and subsequent degradation in the proteasome (16). However, the regulatory effect of M-cadherin on phosphorylation status of β -catenin N-

terminus and the impact of this regulation on myogenic differentiation remains to be elucidated.

The **central hypothesis** of this project is that **M-cadherin-mediated signaling regulates the biological behavior and fate of myoblasts by (i) protecting myoblasts against mitochondrial dysfunction-associated intrinsic apoptosis via the PI3K/Akt/GSK-3 β pathway and (ii) attenuating GSK-3 β -dependent phosphorylation of β -catenin at N-terminus and maintaining a signaling-active unphosphorylated β -catenin, which is critical to canonical Wnt signaling.**

This hypothesis will be tested by the following specific aims.

Specific Aim 1: To determine if M-cadherin-mediated signaling may protect C2C12 myoblasts against apoptosis and if this protective effect is mediated by the PI3K/Akt/GSK-3 β pathway.

Hypothesis 1.1: Knockdown of M-cadherin expression by RNAi will sensitize myoblasts to mitochondria-associated intrinsic apoptosis induced by either cell confluence or serum starvation while activation of M-cadherin-mediated signaling has the opposite effect, indicating that M-cadherin protects myoblast against apoptosis.

Hypothesis 1.2: Knockdown of M-cadherin expression by RNAi will decrease Akt activation and attenuate the Akt-dependent inhibitory phosphorylation of GSK-3 β . Conversely activation of M-cadherin-mediated signaling will have the opposite effect. In addition, the GSK-3 β inhibition by M-cadherin is PI3K dependent.

Hypothesis 1.3: Overexpression of GSK-3 β will sensitize myoblasts to apoptosis induced by either cell confluence or serum starvation while inhibition of GSK-3 β has the opposite effect, indicating activation of GSK-3 β promotes apoptosis of myoblasts.

Hypothesis 1.4: Inhibition of GSK-3 β activity will rescue the apoptotic and myogenic phenotype of myoblast caused by M-cadherin RNAi, indicating the protective effect against apoptosis by M-cadherin is mediated by GSK-3 β suppression.

Specific Aim 2: To determine if M-cadherin-mediated signaling may protect primary muscle progenitor cells against apoptosis and if this protective effect is mediated by GSK-3 β activity.

Hypothesis 2.1: Knockdown of M-cadherin expression by RNAi in primary progenitor cells will exacerbate apoptosis and impair the myogenic differentiation in specific population of primary progenitor cells.

Hypothesis 2.2: Inhibition of GSK-3 β activity by certain chemical inhibitors will rescue the apoptotic and myogenic phenotype caused by M-cadherin RNAi in primary muscle progenitor cells;

Specific Aim 3: To determine if M-cadherin modulates phosphorylation status of β -catenin N-terminus and the effect of this modulation on myoblast fate specification.

Hypothesis 3.1: Knockdown of M-cadherin expression by RNAi will enhance phosphorylation of β -catenin N-terminus by GSK-3 β while inhibition of GSK-3 β by a chemical inhibitor will rescue this effect, indicating that M-cadherin regulates phosphorylation status of β -catenin at N-terminus in a GSK-3 β activity-dependent manner.

Hypothesis 3.2: Chemical inhibition GSK-3 β will promote myogenic differentiation while M-cadherin RNAi will abrogate this effect, indicating M-cadherin is required for GSK-3 β inhibition-induced increase in myogenic differentiation.

Hypothesis 3.3: β -catenin RNAi or treatment with canonical Wnt inhibitor will effectively reverse the myogenic induction caused by chemical inhibition of GSK-3 β , indicating the phenotype of myoblasts induced by GSK-3 β inhibition is mediated by β -catenin/canonical Wnt signaling.

Hypothesis 3.4: Transfection of a phosphorylation-resistant mutant form of β -catenin will rescue the myoblast phenotype caused by M-cadherin RNAi, indicating that M-cadherin's role in promoting myogenic differentiation is at least partially mediated by the modulation of the phosphorylation status of β -catenin N-terminus.

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CHAPTER 1:
BACKGROUND AND SIGNIFICANCE

1.1. Satellite cells and muscle regeneration

1.1.1. Satellite cells: heterogeneity and molecular markers. Satellite cells were first identified in 1961 by Mauro (1). They are named after their typical position in muscles that is between the basal lamina and the sarcolemma of their associated muscle fibers. Satellite cells are considered as the primary source of stem cells for adult muscle regeneration and plays a crucial role in mediating the postnatal growth of muscle (2). Satellite cells are heterogeneous in their embryological origin, fiber association, and biological behavior when culture in vitro (3-5). Several markers have been used to identify the majority of satellite cells, including syndecan-3 and -4, integrin- α 7, CD34, M-cadherin, c-met, Pax7, Myf5, etc (4). Many groups have separated different subpopulations of satellite cells using FACS (fluorescence-activated cell sorting) based on the expression of surface markers. For example, the Olwin lab reported that syndecan-4 is a reliable marker for muscle-derived stem cells (6). Wagers' group showed that a population of satellite cells that are CD45⁻Sca1⁻Mac1⁻CXCR4⁺ β 1integrin⁺ are more efficient in engrafting into dystrophic muscles compared with control populations without this marker combination when both transplanted into host muscles (7).

1.1.2. Activation, proliferation and differentiation of satellite cells. In the adult muscle, under normal physiological resting state, satellite cells are quiescent both mitotically and metabolically. In response to stress stimuli such as muscle injury or increased workload, the quiescent satellite cells can be activated and move out of their niche to the basal lamina and re-enter the cell cycle with Pax7 and Myf5 expressed. The descendents of activated satellite cells are defined as

myoblasts that will proliferate and undergo multiple divisions and differentiate and fuse to form multinucleated myofibers, which is a critical process for post-injury muscle regeneration (8; 9). The mechanism that explains how satellite cells are activated in response to a range of stimuli remains largely unknown. Various growth factors and secondary messengers, such as hepatocyte growth factor (HGF) (10), fibroblast growth factor (FGF) (11; 12) and nitric oxide (NO) (13) are thought to play important roles in the activation of satellite cells. After being activated, muscle regulatory factors (MRFs) are expressed in satellite cells at different stages of myogenesis (14): Myf5 is expressed in both quiescent and activated satellite cells and proliferating myoblasts. MyoD expression start as the satellite cell is activated. The expression of myogenin occurs in myoblasts that exit cell cycle and marks the first commitment step for myogenic differentiation. As the cell cycle inhibitor p21 accumulates in the nuclei, terminal differentiation is accomplished with the expression of contractile and associated proteins such as myosin heavy chain (MyHC) and Troponin T (15). The muscle cells fuse to each other to form myotubes in vitro or myofibers in vivo (16; 17) (Figure 1).

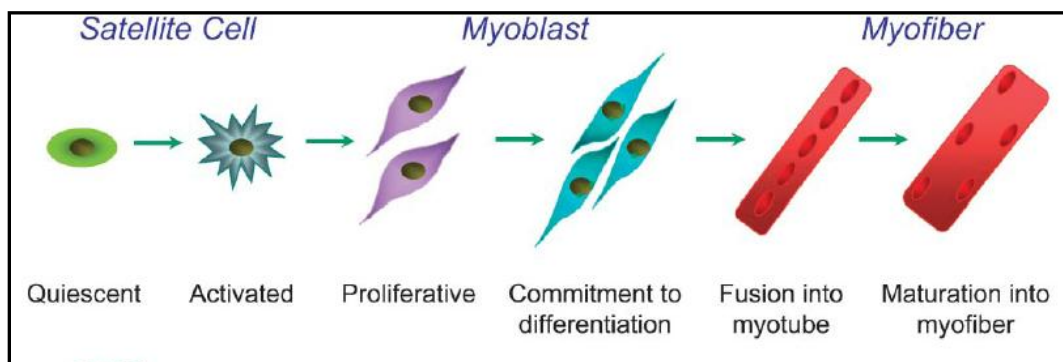


Figure 1. Schematic process of satellite cell myogenesis (14).

The C2C12 cell line is a subclone (produced by H. Blau, (18)) of the mouse myoblast cell line established by D. Yaffe and O. Saxel (19). When being cultured in vitro in growth medium at a low cell density, the C2C12 myoblasts are capable of proliferating for self-renewal. When they obtain confluence or when they are cultured in low-serum conditions, they differentiate spontaneously and rapidly, fuse with each other, and form contractile myotubes and produce characteristic muscle proteins. Thus, the C2C12 myoblast is an established and well-studied in vitro model for activated myoblasts (18).

1.1.3. Satellite cell dysfunction-related muscular dystrophic disorders. In mouse, fast myofibers contain about 300 myonuclei and they are associated with 5-12 satellite cells per fiber. In a slow myofibers, there are about 450 myonuclei associated with about 30 satellite cells (20). Studies have demonstrated that satellite cells undergo an asymmetrical division, which generates a replacement satellite cell and a daughter cell that is committed to a myogenic fate (21). Thus, satellite cells may maintain a constant pool during the process of muscle regeneration (22). The total number of satellite cells decreases in both fast and slow muscle fibers with aging in rodents. At birth, satellite cells account for about 32% of total muscle nuclei, but the number drops to less than 5% in adult muscle (23). A significant decline in the number of M-cadherin-positive satellite cells in human muscle during aging was also reported (24). Interestingly the proliferation and regenerative capacity of satellite cells in old skeletal muscle could be restored when exposed to a young systemic environment by parabiotic pairings of young and old animals, indicating that in addition to intrinsic potential of the cells, the

extrinsic microenvironment, the niche where satellite cells reside is very important in regulating the functional status of satellite cells (25; 26).

The impaired capability of proliferation and differentiation of satellite cells is regarded as one of the major factors that contribute to the occurrence and progression of certain muscle wasting conditions, such as sarcopenia and Duchenne Muscular Dystrophy. Sarcopenia is a Greek term that means lack of flesh. This is an aging-associated decline of muscle mass, quality and performance which leads to frailty in the elderly (27; 28). Sarcopenia begins in the fourth decade of life and accelerates after the age of 70. The total cross section area of skeletal muscle decreases by up to 40% at the age of 80s compared with that at the age of 20s (29). Sarcopenia has multiple detrimental effects and leads to physical disability, loss of independence, a variety of metabolic disorders and increased morbidities. The estimated health care costs directly related to sarcopenia was about 18.5 billion dollars in US in 2000 (30). Both number and functionality of satellite cells decline in aged people with sarcopenia (31; 32). Another pathological condition that is associated with decreased or even exhausted number and functionality of satellite cells is Duchenne Muscular Dystrophy (DMD), which is a lethal recessive x-linked muscular dystrophy. The mutation occurs in the gene coding for dystrophin. Dystrophin is a cytoplasmic protein, a vital component of dystroglycan complex (DGC) which connects the cytoskeleton of a muscle fiber to the surrounding extracellular matrix (ECM) via cell membrane. Thus the DGC is critical for cell integrity, signaling and muscle function. Without DGC, the muscles undergo repeated injury and regeneration due to the mechanical stress during

contraction, which results in rapid exhaustion of satellite cells and rapid progression of muscle degeneration, eventually leads to loss of ambulation, paralysis and death. DMD is one of the most prevalent muscular dystrophies and affects one out of 3500 males in USA (33).

1.2. β -catenin/canonical Wnt signaling and its role in muscle regeneration.

1.2.1. β -catenin/canonical Wnt signaling and its regulation by GSK-3 β .

Wnts are secreted extracellular proteins that trigger a wide range of cellular responses upon receptor binding and activation (34). Wnt signaling can be broadly categorized as canonical or noncanonical pathways. Both require the Wnt ligation of Frizzled receptor (Figure 2). But the noncanonical Wnt pathway is β -catenin-independent, mediated by the intracellular calcium ion, JNK, and PKC, which leads to signaling events such as activation of NFAT transcription and planar cell polarity (35), which is not the focus of this review. Canonical Wnt pathway is initiated by the binding of Wnts to two cell surface proteins, one is the Frizzled receptor, the other is lipoprotein receptor-related protein-5 and 6 (LRP5/6). Ligation of Wnts triggers phosphorylation of LRP5/6's cytoplasmic tail and recruits Disheveled protein to the plasma membrane, followed by recruitment of Axin and GSK-3 β to Disheveled, which inactivates the so-called destruction complex for β -catenin (36). β -catenin belongs to the armadillo family of proteins, which have a central domain consisting a repeating 42 amino acid motif termed as the "arm repeat" (37). X-ray crystallographic analysis shows that the 12 arm repeats in the central domain of β -

catenin form a superhelix of helices. This creates a long, positively charged groove (38) that is capable of interacting with many negatively charged ligands, such as cadherin, the Axin/APC destruction complex, and the TCF/LEF (T cell factor/lymphoid enhancer factor) transcription factors (Figure 3). In the absence of Wnt ligands, cytosolic β -catenin is recruited to a destruction complex composed of Axin, adenomatous polyposis coli (APC), and Glycogen Synthase Kinase-3 β (GSK-3 β). The N-terminus of β -catenin is phosphorylated sequentially by axin-bound casein kinase I α (CK1 α) at serine 45, and by GSK-3 β at serine 33/37 and threonine 41 (39) (Figure 2 and Figure 3). The N-terminal phosphorylated β -catenin is recognized by β -TrCP (β -transducin repeat-containing protein), a component of the E3 ubiquitin ligase complex, which results in its rapid degradation in 26S proteasome (40). So without Wnt signaling, the level of β -catenin is kept low by degradation of cytoplasmic β -catenin. Thus, the transcription factors LEF1 and TCF interact with Grouchos in the nucleus to repress Wnt-specific transcription. In the presence of Wnt ligands, LRP5/6 surface receptors are phosphorylated by CKI γ and GSK-3 β , Dishevelled is recruited to the plasma membrane which leads to dissociation of the destruction complex, resulting in stabilization and accumulation of cytosolic β -catenin. β -catenin translocates into nucleus and displaces Grouchos and forms a transcriptionally active complex. The TCF/LEF transcription factors then induce Wnt-specific target gene expression (36) (Figure 2). Therefore, the key regulatory factor in β -catenin signaling is its stabilization and accumulation in the cytosol (41-43).

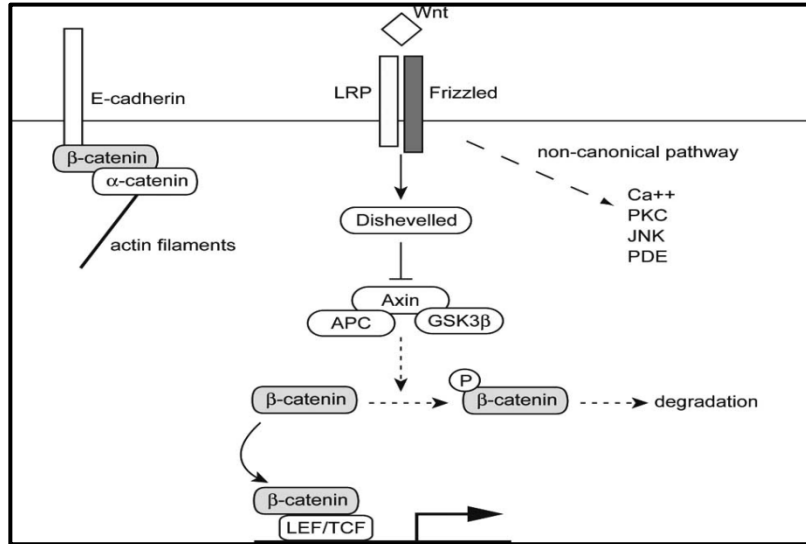


Figure 2. Dual role of β-catenin in cadherin and canonical Wnt signaling pathways (erbiotek.erciyes.edu.tr/dosyalar/Molecular_mechanisms_of_pluripotency.ppt)

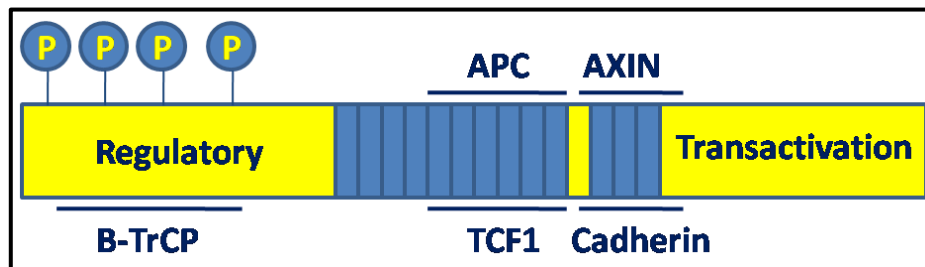


Figure 3. Regulatory residues at N-terminus and binding sites in β-catenin protein.

1.2.2. The role of β -catenin/canonical Wnt signaling in regulating muscle regeneration. Canonical Wnt signaling regulates many cellular events including proliferation, differentiation, and morphogenesis. Dysregulated Wnt signaling has been associated with cancers especially colon cancers, tissue fibrosis, osteoporosis, etc (34; 36). In skeletal muscle, it was demonstrated that in response to muscle injury, the muscle resident CD45+ stem cells were mobilized to enter cell cycle for self-expansion. Furthermore, activation of Wnt signaling effectively induces the myogenic specification of these muscle resident CD45+ stem cells (44). It was also shown that activation of β -catenin signaling induces myogenesis and inhibits adipogenesis in mesenchymal stromal cells (MSC) (45). The expression of β -catenin and activation of Wnt signaling in activated satellite cells promotes their proliferation and self-renewal (46; 47). More importantly, by working collaboratively with Notch signaling, Wnt signaling is critical for myogenic differentiation. Upon muscle injury, the expression of Notch signaling components (Delta-1, Notch-1, and active Notch) is up-regulated in activated satellite cells. Increased Notch signaling promotes the transition of activated satellite cells to highly proliferative myoblasts. However, the elevated Notch signaling also prevents the occurrence of myogenic differentiation (48). As the cells start to commit to myogenic differentiation, there is a transition from Notch signaling to Wnt signaling in myogenic precursor cells with an increase in both Wnt expression in tissue and Wnt responsiveness in progenitor cells (49). On the other hand, hyperactivation of Wnt signaling turns myogenic progenitor cells into a fibrotic lineage and results in muscle fibrosis (50).

Recent studies have added novel information to the mechanism of β -catenin signaling and they show more clearly how it regulates myogenesis. It was reported that upon Wnt stimulation, the N-terminal unphosphorylated β -catenin is more responsive, and accumulates in nuclei (51; 52). Additionally, the N-terminal unphosphorylated β -catenin is more signaling-active and plays a major role in mediating canonical Wnt signaling (53). Thus the GSK-3 β -dependent phosphorylation of β -catenin at the N-terminus alone might be sufficient to inhibit β -catenin activity by preventing its nuclear translocation via cytosolic sequestration, without affecting its stability in certain types of cells and tissues. Furthermore, in addition to its classical binding partner in nuclei, TCF/LEF, β -catenin was found to interact with I-mfa (an inhibitor of the MyoD family of muscle MRFs) in muscle progenitor cells. This interaction relieved the transcription activity suppression and cytosolic sequestration of MRFs caused by the binding of I-mfa (54). In addition, β -catenin has been shown to directly bind to MyoD and this binding enhances MyoD transcription activity that is necessary for myogenic differentiation, whereas TCF/LEF activity is dispensable for this effect (55). These findings suggest that β -catenin/canonical Wnt signaling may promote myogenesis independent of TCF/LEF transcription activity.

1.3. Apoptosis and its role in muscle remodeling and satellite cell biology

1.3.1. Apoptosis and its molecular regulation. Apoptosis is a systemic process of programmed cell death that is important for normal tissue morphogenesis and

homeostasis during development. Apoptosis is a genetically predetermined mechanism that may be initiated by three different molecular pathways. Firstly, the extrinsic death receptor-mediated pathway of apoptosis is triggered by the ligand binding-induced activation of death receptors at the cell surface. The death receptors include the tumor necrosis factor (TNF) receptor-1, CD95/Fas (the receptor of CD95L/FasL) and TNF-related apoptosis inducing ligand (TRAIL) receptors-1 and 2. The ligation of death receptors leads to their homo-trimerization and the recruitment and oligomerization of the cytoplasmic adapter molecule FADD (Fas-associating death domain-containing protein) within the death-inducing signaling complex (DISC). Oligomerized FADD binds to caspases-8 and 10, resulting in their dimerization and activation (56). The second pathway is intrinsic mitochondrial-associated pathway which is activated in response to extracellular or intracellular insults such as DNA damage, and characterized by the occurrence of mitochondrial outer membrane permeabilization (MOMP), which is marked by the release of a number of proteins that normally exist within the intermembrane space (IMS) into an extramitochondrial compartment (cytosol and nuclei). Many proteins released from mitochondria including Cyt c (cytochrome c), AIF (apoptosis inducing factor), EndoG (endonuclease), and Smac/DIABLO (second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with a low isoelectric point) are proapoptotic in the cytosol or nucleus (57). Cyt c, a component of the mitochondrial respiratory chain, interacts with its adaptor molecule Apaf-1 (apoptosis protease activating factor 1) in cytosol and triggers the ATP/dATP-dependent oligomerization of Apaf-1. Oligomerized Apaf-1 binds to

procaspase-9, leading to the formation of the caspases-9 activation complex named as apoptosome (58) that cleaves procaspase-9 into its active form. This in turn cleaves and activates the executioner caspases, such as caspases-3 and -7, leading to the cleavage of caspases substrate proteins and resulting in the biological and morphological features of programmed cell death. The third pathway is also intrinsic and associated with endoplasmic reticulum (ER) stress, which includes disruption of ER calcium homeostasis and accumulation of excessive proteins in ER. Caspase-12 is activated by ER stress as an initiator caspase that is responsible to the cascade of executioner caspases activation (59). However, the precise mechanism remains to be elucidated.

1.3.2. Apoptosis in muscle remodeling and satellite cell biology. Dysregulated apoptosis contributes to the occurrence and progression of many pathological conditions including autoimmune diseases, cancer, neurodegenerative diseases, and certain cardiovascular diseases (56; 60). In skeletal muscle, accumulating evidence suggests that apoptosis plays an important role in muscle remodeling. Elevated apoptotic signaling has been detected in various muscle wasting conditions caused by disuse, denervation, and aging (61-63). As for the satellite cells, their number in aged muscle declines due to increased apoptotic depletion in aged muscle (64). Moreover, when cultured in vitro, muscle progenitor cells isolated from aged muscle are more sensitive to apoptotic insults compared with the cells isolated from young muscles (65). Elimination of muscle progenitor cells by apoptosis also contributes to the low regeneration capability of dystrophic muscles and the poor outcomes of stem-cell-based therapeutic strategies (66-69).

In addition, in vitro culture of myoblasts has shown that apoptosis is concurrent with myogenic differentiation (70).

1.4. Cadherin and its role in apoptosis, β -catenin/canonical Wnt signaling and muscle regeneration.

1.4.1. Cadherins and their roles in regulating cell survival. Cadherins are Ca^{2+} -dependent cell adhesion molecules that are the key components of several cell-cell adhesion complexes including adherens junctions and desmosomes. The cadherin superfamily consists of different subfamilies: classic cadherins (type I & type II), desmosomal cadherins, protocadherins, and other related cadherins (71; 72). The classical cadherins are composed of a highly conserved carboxy-terminal cytodomain, a single-pass transmembrane domain, and five extracellular cadherin-motif subdomains (C1-C5) with a conserved His-Ala-Val (HAV) sequence in C1 of type I classic cadherins. They mediate homophilic, Ca^{2+} -dependent cell-cell adhesion. In the absence of calcium, the cadherin structure is disorganized and incapable of participating in adhesion. As calcium concentration increases, the C1-C5 subdomains become organized (C1, which has the lowest affinity for calcium, becomes organized last) and the cadherin ectodomains become rigid and competent to participate in cis-dimerization and also trans-dimerization as the calcium concentration increases (73; 74). In addition, to establishing strong adhesion, classical cadherins need to link to the cytoskeleton. This is mediated by binding of the cytoplasmic tail of classical cadherin to β -catenin, which in turn binds to α -catenin. α -catenin bridges the cadherin- β -catenin complex to the actin

cytoskeleton either directly or indirectly via actin-binding proteins such as α -actinin or profilin, thus coupling ectodomain interactions to dynamic intracellular tensile forces (72).

The accumulating evidence shows that cadherin-mediated cell-cell adhesion provides a survival signal in a variety of cell types, including neural cells (75), endothelial cells (76; 77), ovarian cells (78; 79), granulosa cells (80), enterocytes (81), vascular smooth muscle cells (82) and many tumor cells (83-87). Cadherins mediate cell survival via several possible mechanisms as revealed by studies on different cell types. One of the most important pathways that mediate cadherins' survival-promoting effect is PI3K/Akt, which can be activated by different types of cadherins, and results in the phosphorylation and inactivation of the pro-apoptotic protein Bad, and the stabilization of the anti-apoptotic protein Bcl-2 (80; 85; 87; 88). This cadherin-dependent Akt activation appears to be ligand/growth-factor independent (86-88). Another candidate pathway is fibroblast growth factor receptor (FGF-R) which also has a HAV motif in its extracellular domain. Cadherins like N-cadherin has an IDPVNGQ motif in ECD4 which can bind to the HAV motif of FGF-R. Therefore, by forming heterodimers with FGF-R through the binding of these two motives, cadherins can activate FGF-R and mediate cell survival (76; 89). Cadherin may also regulate cell survival via β -catenin. However, the role of β -catenin in cell survival/death is cell-type specific. β -catenin is survival-promoting in some types of cells including epithelial cells cultured in suspension (90), fibroblasts (91), and an embryonic liver culture system (92), but apoptosis-

promoting in other types of cells, including COS7 and 293 cells (93), as well as several cancer cell lines (94).

1.4.2. The role of cadherins in regulating myogenesis. Cadherins also play an important role in regulating myogenesis. Gurdon et al reported that during myogenesis, the “community effect”, occurs, whereby cells must contact a sufficient number of like neighbors if they are undergoing coordinate differentiation within a developing tissue (95; 96). N-cadherin is expressed throughout the process of avian and murine myogenesis, and antibodies that neutralize N-cadherin inhibit differentiation of myogenic cells (97-99). Conversely, recombinant cadherin ectodomains coupled to planar substrates or beads, which are able to induce lateral clustering of cadherins at sites of attachment, facilitate the recruitment of catenin to cadherin and induce the reorganization of actin cytoskeleton system thus allow myoblasts to differentiate at low cell density, in a similar way to that observed in cells cultured on plastic substrate at high cell density (100; 101). As for the signaling events downstream to cadherins leading to myogenic gene programming, it's reported that cadherin ligation results in up-regulation of p21 and p27 cyclin-dependent kinase inhibitors and cell cycle arrest, and increases expression of myogenin, and muscle-specific sarcomeric proteins. In addition, cadherin ligation can also regulate the reorganization of actin cytoskeleton by altering the activation status of Rho GTPases (activating RhoA but suppressing Rac1 and Cdc42), the established regulators of actin dynamics (99-101).

1.4.3. The role of cadherins in regulating β -catenin transcription activity.

Sadot reported that in Chinese hamster ovary (CHO) and SW480 colon carcinoma cell lines, the binding of β -catenin to the cadherin cytoplasmic tail either in the membrane, or in the nucleus, can inhibit β -catenin degradation and efficiently block its transactivation (102). Gottardi reported that expression of wild-type E-cadherin significantly inhibits the growth of colorectal tumor cell line via inhibiting β -catenin/TCF gene promoter activity in an adhesion-independent manner (103). Similarly, Stockinger demonstrated that forced expression of E-cadherin in both fibroblasts and epithelial cells induces cell cycle arrest by inhibiting β -catenin transcriptional activity (104). Gauthier-Rouviere's group reported that N-cadherin-dependent cell-cell contact activates muscle-specific promoters and RhoA in C2C12 cells. RhoA activity is required for β -catenin recruitment to intercellular adhesions sites (105). Kuphal and Behrens (106) reported that in DLD-1 colorectal cancer cells, inhibition of E-cadherin expression by RNA interference led to nuclear translocation of β -catenin and an enhancement of β -catenin/TCF-dependent reporter activity. On the other hand, forced expression of E-cadherin in L929 fibroblasts which are deficient in both E-cadherin expression and Wnt signaling induces the stabilization of β -catenin at the cell junctions and resulted in evident changes in cell phenotype but no significant impact on the expression level of Wnt-related genes. Recent study from Gottardi lab demonstrated that in S2480 colon carcinoma cell line, the forced expression of E-cadherin enhances the GSK-3 β -dependent phosphorylation of β -catenin N-terminus, leading to the inhibition of Wnt

signaling. This observation provides a novel mechanism underlying cadherin's regulation on β -catenin activity (107).

1.4.4. M-cadherin and its role in myogenesis and apoptosis. M-cadherin is a member of classic cadherins. It was first identified in differentiating muscle cells by Donalies and colleagues (108) in 1991. They reported that M-cadherin mRNA was present at low levels in myoblasts and up-regulated in myotube-forming cells but not detectable in mouse fibroblasts. Its gene locus is linked to the E-cadherin (but not N-cadherin) locus on chromosome 8 of mouse and the human homologue was mapped to chromosome 16q24.1 (109). During the process of myogenesis, M-cadherin is specifically involved in secondary myogenesis and its expression is down-regulated after birth (110-112). In mature skeletal muscle, M-cadherin is only detectable on satellite cells and on the sarcolemma of myofibers underlying satellite cells. At early stages of regeneration, M-cadherin was exclusively and strongly expressed in myoblasts. After fusion of myoblasts into myotubes, M-cadherin was down-regulated and was barely detectable on more mature myotubes surrounded by distinct basal lamina sheaths (113; 114). It's reported that there was a significant decline in the number of M-cadherin-positive satellite cells in human muscle during aging (24).

During the past decade, the M-cadherin's role in myoblast fusion has been established attributed to the studies from various groups. Kuch et al (115) reported that similar as E-cadherin and N-cadherin, M-cadherin formed two distinct cytoplasmic complexes in myogenic cells, either with α -catenin/ β -catenin or with α -catenin/plakoglobin, indicating that M-cadherin shares important features with the

classical cadherins in spite of its phylogenetic divergence. Zeschnigk and coworkers (116) treated myoblasts with antagonistic M-cadherin peptides and found the fusion of myoblasts into myotubes was inhibited and the expression of troponin T, an established marker of terminal myogenic differentiation, was downregulated. The cell cycle withdrawal process was also impaired. Their data strongly suggested that M-cadherin-mediated myoblast interaction plays an important role in terminal differentiation of skeletal muscle cells. Charrasse and coworkers demonstrated that M-cadherin may mediate myoblast fusion by activating Rac1 GTPase and its function is regulated by RhoA which promotes M-cadherin's degradation through lysosomal pathway (117; 118). In spite of the above evidences that establish M-cadherin's role in myogenesis and muscle regeneration, there is another study questioning M-cadherin's absolute necessity in myogenesis and muscle regeneration. Hollnelt and colleagues generated an M-cadherin-null mutation in mice. The mutant mice were viable and fertile and showed no gross developmental defects. The skeletal muscle appeared normal. Moreover, muscle lesions induced by cardiotoxin injection were efficiently repaired in mutant mice, suggesting that satellite cells can be activated to form new myofibers. Thus they concluded that M-cadherin in the mouse doesn't serve absolutely required function during muscle development and regeneration and the lack of M-cadherin seems to be largely compensated by N-cadherin or other cadherins. However, they failed to observe a compensatory increase in either the expression or the function of any other cadherins such as N-cadherin, in M-cadherin^{-/-} skeletal muscle. Furthermore, they didn't demonstrate the status of

regeneration capability of muscle in aged M-cadherin-null mice (119). Thus the importance of M-cadherin in muscle regeneration, as well as the mechanism that how M-cadherin regulates muscle regeneration, remains to be elucidated.

Although the role of other cadherins, (e.g. E-cadherin, N-cadherin, VE-cadherin), in regulating cell survival and death are under extensively investigation, there is no report published concerning M-cadherin' role in regulating the apoptosis of myoblasts or satellite cells. Furthermore, the role of M-cadherin in regulating β -catenin phosphorylation status and the impact of this regulation on canonical Wnt signaling and myogenesis has not been addressed yet.

1.5. PI3K/Akt/GSK-3 β pathway and its role in apoptosis and muscle remodeling

1.5.1. PI3K/Akt pathway and its role in cell survival and apoptosis.

Phosphatidylinositol 3-kinase (PI3K) is a heterodimer composed of a regulatory subunit (p85) and a catalytic (p110) subunit and possesses activities of both phosphoinositide kinase and serine/threonine protein kinase (120; 121). P85 subunit negatively regulates the catalytic activity of the p110 subunit (121). The trigger event for PI3K activation is the activation of receptor or non-receptor tyrosine kinases in response to ligation of growth factors on the plasma membrane, which leads to the autophosphorylation on tyrosine residues and transphosphorylation of adaptor proteins, generating specific phosphotyrosine sequences that bind to the SH2 domain of p85 and alleviates its inhibition on p110. This results in the enzymatic activation of PI3K (122). On the other hand, GTP-associated Ras can induce the activation of PI3K by directly binding to the p110

subunit (123). Once activated, PI3K phosphorylates phosphatidylinositol at the 3'-OH of the inositol ring and converts phosphatidylinositol (3,4,) biphosphate (PIP₂) to phosphatidylinositol (3,4,5) triphosphate (PIP₃) (124; 125). This functions as a lipid second messenger and has a high affinity to the pleckstrin (PH) domain-containing proteins, including the serine/threonine kinase Akt. The binding to PIP₃ recruits Akt to the inner surface of plasma membrane (126). Phosphoinositide dependent kinase 1 (PDK1), another PH domain-containing protein, is also recruited to plasma membrane by PIP₃ (127). Full activation of Akt requires phosphorylation of the Tyrosine308 and Serine473 residues by PDK1 and PDK2 respectively (127-129). Akt is the key downstream target of PI3K that transmits most signals from PI3K. A large number of downstream effectors of Akt have been identified, including mTOR, p70S6K1, Forkhead (FH) transcription factors, GSK-3 β , IRS-1, caspase-9, Bad, and eNOS (130). The PI3K/Akt pathway has been established as one of the key signaling pathways that play a crucial role in regulating various cellular processes, such as cell proliferation, survival, differentiation, protein synthesis, glucose metabolism, and cell motility (129; 130). Akt is also known as protein kinase B (PKB). It was originally identified as the oncogene in the transforming retrovirus, AKT8, which was isolated from an AKR mouse thymoma cell line (131). AKT plays a crucial role in cellular survival pathways by phosphorylating a number of target proteins. Akt directly phosphorylates human procaspase-9 at serine196 and inhibits its protease activity (132). Akt also phosphorylates Bad, a proapoptotic member of Bcl-2 family, at the residue of serine136. This phosphorylation releases Bad from the complex with

Bcl-2/Bcl-XL localized on the mitochondrial membrane and promotes its binding to 14-3-3 proteins in the cytosol, leading to the inhibition of its proapoptotic activity (133). In addition, Akt is able to phosphorylate Forkhead (FoxO) transcription factors and this phosphorylation leads to their nuclear exclusion and the suppression of FoxO-related proapoptotic transcription events (134-136). Akt also activates IKK α (I κ B kinase α) and promotes NF- κ B-mediated survival signaling (137). Furthermore, Akt phosphorylates Glycogen Synthase Kinase-3 β (GSK-3 β) at the N-terminal regulatory residue of serine 9, and this phosphorylation inactivates GSK-3 β (138).

1.5.2. GSK-3 β : function, regulation, and its role in apoptosis. GSK-3 β is a serine/threonine kinase that phosphorylates numerous substrates including transcription factors, structural proteins, and signaling proteins. It is involved in various physiological pathways such as glucose homeostasis and metabolism, cell cycle control, cell morphogenesis, development, tumor transformation, and neuroprotection. Thus it has been associated with many diseases including type II diabetes, inflammation, cancer, Alzheimer's disease, and bipolar disorder (139; 140). Since GSK-3 β is constitutively active in cells under normal resting conditions, the primary regulatory mode of GSK-3 β activity is to inactivate it, which is achieved by phosphorylation of an N-terminal residue of serine 9 which creates a pseudosubstrate that occupies the active site of the enzyme, preventing its interaction with real substrates (141). The kinases that are able to inactivate GSK-3 β include PKA (cyclic AMP-dependent protein kinase) (142), PKB/AKT (protein kinase B)(138), PKC (protein kinase C) (143), and p90RSK (p90 ribosomal S6

kinase) (144). In addition, there are more than 30 chemical compounds that have been identified to be the GSK-3 inhibitors (145). Lithium chloride (LiCl) is one of the earliest established inhibitors for GSK-3. LiCl inhibits GSK-3 activity by two mechanisms: firstly, it competes with ATP in the ATP-binding site of the kinase via directly competing with magnesium; secondly, it increases the inhibitory phosphorylation of Serine 9 residue of GSK-3 β and Serine 21 residue of GSK-3 α (146). The small heterocyclic thiadiazolidinones (TDZD) are the first group of chemicals that inhibit GSK-3 β activity in a non-ATP-competitive pattern (147).

GSK-3 β is an established negative regulator of canonical Wnt signaling by phosphorylating β -catenin (39). GSK-3 β also plays an important but paradoxical role in regulating apoptosis. Firstly, it promotes intrinsic mitochondria-associated apoptosis (148). GSK-3 β can directly phosphorylate Bax at Serine163 which leads to Bax activation (149). GSK-3 β promotes p53-induced intrinsic apoptosis by both upregulating its expression level and regulating its intracellular localization (150; 151). GSK-3 β can also phosphorylate and inhibit a number of transcription factors that favor the gene expression for anti-apoptotic proteins. For example, the β -catenin/canonical Wnt signaling pathway has been shown to promote growth and survival. However, phosphorylation of β -catenin by GSK-3 β results in its degradation and the turnoff of canonical Wnt signaling (39). Another survival-promoting transcription factor that is inhibited by GSK-3 β is HSF-1 (Heat shock factor-1), which induces the expression of several heat shock proteins that protect cells against apoptosis (152-154). Overexpression of GSK-3 β has been shown to induce apoptosis in various types of cells, including PC12 cell and Rat1 fibroblasts

(155), cortical neurons (156), vascular smooth muscle cells (157), endothelial cells (158), and human umbilical vein endothelial cells (159). On the other hand, GSK-3 β antagonizes the death receptor-mediated extrinsic apoptosis (148). The mouse GSK-3 β knockout model results in embryonic lethality because of massive liver apoptosis due to TNF hypersensitivity (160), indicating that GSK-3 β inhibits TNF-induced apoptosis. Although the exact molecular mechanism that underlies the anti-apoptotic effect of GSK-3 β in extrinsic apoptosis remains to be elucidated, accumulating evidences suggest that GSK-3 β -dependent anti-apoptotic effect is executed at a very early stage that precedes the initiating step of caspases-8 activation (148).

1.5.3. The role of GSK-3 β in muscle wasting and satellite cell biology. Many studies have suggested that GSK-3 β negatively regulates cardiac hypertrophy and inactivation of GSK-3 β is an important mechanism in the development of cardiac hypertrophy (161; 162). In skeletal muscles, association between GSK-3 β and various muscle wasting conditions has been reported in many studies. GSK-3 β activity is increased in aged (163) and burn-injured skeletal muscles (164). The activity of GSK-3 β is downregulated during myogenic differentiation induced by IGF-1 (165). In addition, GSK-3 β inhibition reduces protein degradation in wasting muscles and promotes re-growth of atrophic muscle (166-168). A conditional skeletal muscle knockout of GSK-3 β shows an improved insulin tolerance and glucose metabolism in muscle tissues (169), but there is no data that evaluate a direct role of GSK-3 β in regulating the muscle progenitor cell survival and apoptosis during myogenic differentiation.

Regulation of GSK-3 β activity has been shown to be a key event in mediating the switch between Notch and Wnt signaling as the muscle progenitor cells stop proliferation and start to commit to myogenic differentiation (49), suggesting that GSK-3 β may play a crucial role in regulating the fate specification of satellite cells.

1.6. Summary and relevance.

Number and functionality of satellite cells is crucial to adult muscle regeneration. Understanding the molecular mechanism that underlies the regulation of the survival and differentiation of satellite cells is critical to provide applicable information to potential clinical intervention of certain muscular dystrophic disorders. M-cadherin as a key component for cell-cell adhesions between myoblasts, its function in regulating the survival/apoptosis of myoblasts and the underlying pathways, as well as its role in regulating β -catenin/canonical Wnt signaling have not been addressed. In this dissertation, we tried to seek answers to the following questions: Does M-cadherin protect myoblasts against apoptosis or makes the myoblasts more susceptible to apoptosis, and what is the signaling pathway that mediates this effect? Does M-cadherin regulate phosphorylation status of β -catenin N-terminus in myoblasts? What is the impact of this regulation on canonical Wnt signaling and myoblast biological behavior? Our ultimate goal is to determine whether M-cadherin plays a critical role in regulating myoblasts biological behavior and fate specification and to get insights in novel molecular mechanism of muscle regeneration and to provide clues for novel therapeutic strategies for muscular dystrophic disorders.

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CHAPTER 2:

SUPPRESSION OF GSK-3 β ACTIVATION BY M-CADHERIN PROTECTS MYOBLASTS AGAINST MITOCHONDRIA-ASSOCIATED APOPTOSIS DURING MYOGENIC DIFFERENTIATION

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ABSTRACT

Apoptosis occurs concurrently with differentiation of muscle progenitor cells (MPCs) before they fuse to form myotubes. Dysregulated apoptosis in MPCs contributes to the low regeneration capability in aged muscle and decreases the survival rate of donor cells in stem cell-based therapies including muscular dystrophies. This study investigated the role of the M-cadherin/PI3K/Akt/GSK-3 β signaling pathway in regulating apoptosis during differentiation of MPCs. Disruption of M-cadherin-dependent cell-cell adhesion by M-cadherin RNA interference (RNAi) in confluent C2C12 myoblasts sensitized the cells to mitochondria-associated intrinsic apoptosis induced by cell confluence or serum starvation. Further investigation of this pathway revealed that M-cadherin-mediated signaling suppressed GSK-3 β activation by enhancing the PI3K/AKT-dependent inhibitory phosphorylation of GSK-3 β^{ser9} . Overexpression of wild-type GSK-3 β in confluent C2C12 myoblasts exacerbated the apoptosis, while chemical inhibition of GSK-3 β (TDZD-8), or forced expression of constitutively active Akt (myrAkt), or a kinase-deficient GSK-3 β (GSK-3 β K85R) mutant, attenuated apoptosis and rescued the impaired myogenic differentiation that is caused by M-cadherin RNAi. These data suggest that M-cadherin-mediated signaling prevents acceleration of mitochondria-associated intrinsic apoptosis in MPCs via suppressing GSK-3 β activation during myogenic differentiation.

INTRODUCTION

Muscle progenitor cells (MPCs, i.e., satellite cells) remain quiescent both metabolically and mitotically in adult muscles under normal basal physiological conditions. Once activated by stimuli such as muscle injury or exercise, they enter the cell cycle, proliferate, differentiate, and fuse into myotubes for muscle regeneration (1; 2). The differentiation of MPCs is critical for myotube formation but it also occurs concurrently with apoptosis (3; 4). Apoptosis is a systematic process of programmed cell death which is important for normal tissue morphogenesis and development by maintaining the tissue homeostasis. The dysregulation of apoptosis contributes to a variety of pathologies, including cancer, autoimmune diseases, cardiovascular disease and degenerative neurological diseases (5-7). In skeletal muscle, apoptosis has been linked to conditions of muscle wasting caused by disuse, denervation, and aging (8-10). In addition, inappropriate apoptosis of muscle progenitor cells may contribute to the low regeneration capability of dystrophic muscles and the poor outcomes of stem-cell-based therapeutic strategies (11-14). Decreased muscle progenitor cells number (15), function (16; 17), and altered responses to their niche (18; 19) contribute to the impaired regenerative capability in aging skeletal muscle. In addition, muscle progenitor cells that are isolated from aged muscle are susceptible to apoptosis and their number declines as more of them are depleted by apoptosis in aged muscle (20-22).

M-cadherin is a member of the classical cadherin family of transmembrane glycoproteins mediating calcium-dependent homophilic cell-cell adhesion. M-cadherin is specifically expressed in skeletal muscle and certain neural tissues. In mature skeletal

muscle, M-cadherin is only detectable on satellite cells and the underlying sarcolemma of myofibers (23; 24). The number of M-cadherin-positive satellite cells decreases in aged muscle (25). Although a mouse knockout model indicated that M-cadherin might play a dispensable role in myogenesis and muscle regeneration *in vivo* (26), data from *in vitro* studies showed that by interacting with Rac1 and other members of Rho subfamily, M-cadherin is critical in mediating myoblast alignment and fusion into myotubes (27-29). However, its role in regulating the survival and death of muscle progenitor cells/myoblasts has never been addressed.

In the present study, we investigated the role of M-cadherin-dependent cell-cell adhesion on the survival of mouse C2C12 myoblasts as well as primary muscle progenitor cells during myogenic differentiation. We were particularly interested in the potential for M-cadherin to protect against the mitochondria-associated intrinsic apoptosis that is induced by cell confluence or serum starvation. By disrupting M-cadherin-dependent cell-cell adhesion through knocking down M-cadherin expression via RNA interference, we demonstrated that M-cadherin-mediated signaling is important for maintaining mitochondrial integrity. This occurred by suppressing GSK-3 β activation in a PI3K/Akt-dependent manner, and reducing apoptotic signaling through the mitochondrial pathway, thus promoting the survival of myoblasts during myogenic differentiation. Moreover, apoptosis and impaired myogenic differentiation that is caused by M-cadherin RNAi can be rescued by the inhibition of GSK-3 β activation. Together, these results suggest an indispensable role of M-cadherin-mediated signaling in maintaining the balance between apoptosis and differentiation of muscle progenitor

cells during myogenesis and potentially during activation of muscle stem cells such as that occurring during muscle regeneration.

MATERIALS AND METHODS

For detailed methods see Supplementary information.

Cell Culture

C2C12 myoblasts were purchased from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco Modified Eagle's Medium (DMEM) (Invitrogen Life Technologies, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic and antimycotic mixture solution (Invitrogen). Primary myoblasts were isolated from hindlimb muscles of one-week old C57BL/6 mice (30) and purified by Percoll (Sigma-Aldrich Co., St Louis, MO) gradient centrifugation. The Syndecan-4-positive myoblasts were identified by fluorescence activated cell sorting (FACS) using a Syndecan-4 antibody (BD). The Syndecan-4-positive myoblasts were cultured in Ham's F10 supplemented with 20% FBS at 37°C. Serum starvation was induced by incubating the cells in serum-free DMEM supplemented with 1% antibiotic and antimycotics. Myogenic differentiation was induced by culturing the confluent cells in differentiation medium (DMEM, 2% horse serum, 1% antibiotic and antimycotic).

Chemical Inhibitors

The cells were treated with a PI3K inhibitor Wortmannin (200 nM, Alexis Biochemicals, Plymouth Meeting, PA), a GSK-3 inhibitor, TDZD-8 (25 μ M, Sigma), or a MEK1 inhibitor PD98059 (50 μ M, Cell Signaling, Danvers, MA).

Inhibition of M-cadherin expression by RNA interference

The myoblasts were seeded at a density of 1.7×10^5 per well in a six-well plate, 24 hours before transfection with *SMART*pool small interfering RNA (siRNA) targeted to M-cadherin mRNA (Life Thermo). The transfection media included either Dharma*FECT*TM-3 reagent (Life Thermo) (for C2C12 myoblasts) or Lipofectamine 2000 (Invitrogen) (for primary myoblasts) at a final siRNA duplex concentration of 100nM. The efficacy of M-cadherin protein knockdown by RNA interference (RNAi) was confirmed by immunoblotting.

Plasmids and Transfection

The full-length mouse wild-type GSK-3 β cDNA was generated by RT-PCR. mRNA was derived from wild C2C12 myoblasts using Trizol reagent (Life Technologies, Inc.) and reverse-transcribed into cDNA using Superscript II (Life Technologies, Inc.). The PCR product was cloned into the expression vector pcDNA3.1/myc-His(-) (Invitrogen). The constitutively active mutant of Akt (myrAkt) and the V5-tagged kinase-deficient GSK-3 β mutant (K85R) carried by vector pcDNA3 were generous gifts from Dr. Jia Luo. The cells were transfected with the wild-type GSK-3 β plasmids using FuGENE 6 (Roche Diagnostics, Indianapolis, IN). The co-transfection of M-cadherin siRNA with myrAkt or

GSK-3 β (K85R) plasmids was carried out using DharmaFECT Duo transfection reagent (Dharmacon, Inc., Thermo-Fisher Scientific, Lafayette CO).

Recombinant M-cadherin-Fc treatment

The recombinant M-cadherin-Fc chimera was purchased from R&D Systems (Minneapolis, MN). 6-well plates were pre-coated with goat anti-Fc antibody (0.5 μ g/cm² in PBS/Ca²⁺; Jackson ImmunoResearch, West Grove, PA) overnight at 4°C followed by coating with recombinant M-cadherin-Fc chimera at a final concentration of 2 μ g/cm² in 0.1%BSA/PBS/Ca²⁺ for 2 hours at room temperature. The dishes were blocked with 1% BSA/HBSS/Ca²⁺. Plates coated with solely the anti-Fc antibody were used as vehicle controls. The cells were seeded at 0.5 x 10⁵ cells per well and grown for 48 hours before being harvested for further assays.

Subcellular fractionation

The membrane fraction of C2C12 myoblasts was prepared using a commercial reagent (Thermo Fisher). The nuclear protein fraction was prepared according to methods that have been previously described (31). The mitochondrial, mitochondria-free and nuclei-free cytosolic fractions were prepared using mitochondria/cytosol reagents (BioVision, Mountain View, CA). The concentration of the protein extracts was quantified in duplicate by Bio-Rad *DC* Protein Assay (BioRad, Hercules, CA). The whole cell lysate was obtained by disrupting the cells with RIPA buffer supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, 1:100 dilution) followed by centrifugation. The supernatant was collected as the whole cell lysate.

Immunoblotting

Antibodies specific to phosphoserine 473 Akt, total Akt, phosphoserine 9 GSK3 β , total GSK3 β , cytochrome c, cleaved caspase-9 and cleaved caspase-3, AIF, survivin (1:1000) and cyclin D1 (1:2000 dilution) were purchased from Cell Signaling Technology (Danvers, MA). The anti-M-cadherin antibody (1:200) was obtained from Calbiochem (La Jolla, CA). The anti-caspase-8 antibody (1:250) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-histone H2B (1:5000), β -tubulin (1:500) and -GAPDH (1:5000) antibodies were obtained from Abcam (Cambridge, MA). The antibodies to manganese superoxide dismutase (MnSOD) and copper-zinc superoxide dismutase (Cu-ZnSOD) (1:1000) were purchased from Millipore (Billerica, MA). The secondary antibodies for immunoblotting including goat anti-rabbit or goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The proteins were separated on a 4-12% gradient polyacrylamide gel (Invitrogen), and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were probed with primary antibodies overnight at 4°C, followed by incubation of the appropriate secondary antibody for 1 hour at room temperature. The resulting signals were developed using an enhanced chemiluminescence lighting (ECL) Western blotting detection reagent kit (GE Health Care, Piscataway, NJ). Digital records were obtained from each blot and the protein bands of interest were quantified using 1-D analysis software (Eastman Kodak). The membranes were stripped and reprobed for β -tubulin, GAPDH, or Histone H2B as loading controls.

Immunoprecipitation

The cells were washed in ice-cold PBS and lysed in ice-cold buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.25% SDS, 0.1% Nonidet P-40). Non-soluble materials were removed by centrifugation at 12,000 g. The lysate was incubated with anti-PI3K-p85 α (Abcam), anti-M-cadherin (Santa Cruz) or IgG (Millipore) overnight at 4°C. The sample was then incubated with Protein A/G PLUS-agarose beads (Santa Cruz) and the beads were then collected by centrifugation. The bound proteins were eluted from the agarose beads in Laemmli sample buffer (5X) at 95-100°C. The samples were clarified by centrifugation and the supernatants were separated by SDS-PAGE and immunoblotted against M-cadherin or PI3K-p85 α .

Cell imaging

Phase contrast images of live C2C12 and primary myoblast cells were obtained by a Nikon eclipse TS100 phase-contrast microscope. The digital images were obtained with a SPOT RT camera and analyzed with SPOT RT software (Diagnostic Instruments Inc., Sterling Heights, MI). Immunocytochemical assays were conducted on fixed cells after they had been grown coverslips. After fixation in 4% paraformaldehyde, the cells were incubated at 4°C with antibodies against anti-M-cadherin (1:20, Calbiochem) or anti-myosin heavy chain (MyHC) (1:500, Developmental Studies Hybridoma Bank, Iowa City, IA). The cells were incubated with the Alexa Fluor 546 IgG (H+L) (Invitrogen) and counter-stained with 4,6-diamidino-2-phenylindole (DAPI). The cells were imaged with a Zeiss LSM510 confocal laser scanning microscope using AIM software (Carl Zeiss MicroImaging).

Myoblast fusion index. The myoblast fusion index was calculated as the ratio of the number of DAPI-positive nuclei located in the MyHC-positive myotubes (i.e., fused myoblasts) divided by the total number of nuclei in the same field. This fusion index was used as a read-out of myogenic differentiation. The fusion index was obtained from 10 non-overlapping areas of each coverslip.

Apoptosis assays

DNA fragmentation. DNA fragmentation was used to assess the level of apoptosis in muscle cells using an ELISA (Roche) with measurements for DNA fragmentation that were made at an absorbance of 405nm (31; 32). The data were normalized to the protein concentration of the sample.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). A TUNEL assay (Roche) was used to identify the extent of apoptotic nuclei in adherent myoblasts as reported previously (33). The nuclei of all cells were counter-stained with DAPI. The number of TUNEL- and DAPI-positive nuclei was counted in ten images from non-overlapping areas of each group of cells. The data were presented as the apoptosis index, which was determined by the ratio of TUNEL-positive nuclei to the total number of DAPI-positive nuclei. To identify the full extent of apoptosis, and determine if M-cadherin-RNAi treatment increased the sensitivity of C2C12 myoblasts to serum starvation-induced apoptosis, we assessed apoptosis by a TUNEL assay in both adherent and floating cells in each well using a published method (4). The total percent

of apoptotic cells for each well was calculated as: (the total number of TUNEL-positive attached cells + the total number of TUNEL-positive floating cells) / (total attached cells + total floating cells).

Cardiolipin content and mitochondrial membrane potential measurement

Nonyl acridine orange (NAO, Invitrogen) was used to determine the cardiolipin content in the inner mitochondrial membrane as an indicator of mitochondrial integrity. C2C12 myoblasts were transfected with M-cadherin-targeted (M-) or scrambled siRNA (siCON) with NAO (250nM) at 37°C. Fluorescence was visualized with a Nikon eclipse E800 fluorescence microscope and digital images were captured using a SPOT RT camera (Diagnostic Instruments, Inc). The fluorescence intensity was analyzed and quantified using the ImageJ software (NIH).

To measure changes in the mitochondrial membrane potential, mitochondria were isolated from M-cadherin RNAi or control cells. The purity of mitochondria was verified by staining with MitoTracker Deep Red 633 (Molecular Probes, Carlsbad, CA), a mitochondria-specific marker and analyzed by flow cytometry. This fluorescent dye diffuses passively into intact and respiring mitochondria. The mitochondrial membrane potential ($\Delta\psi_{mt}$) was estimated by staining the mitochondria with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) (Molecular Probes). The staining results of JC-1 orange/green were analyzed using a FACSCalibur system (BD Bioscience) using Cell Quest Pro. 4.0 Software. 100,000 gated events were collected for each sample.

Statistical analyses

The results are presented as mean \pm standard error of mean (SE). Statistical analyses were performed using the SPSS 13.0 software package. A one-way analysis of variance (ANOVA) was used to compare differences in all measured variables. $P < 0.05$ was considered statistical significant.

RESULTS

M-cadherin RNA interference exacerbates mitochondria-associated apoptosis in confluent C2C12 myoblasts. To evaluate the apoptotic propensity to low and high cell densities, C2C12 myoblasts were seeded in 6-well plates at either $2.0 \times 10^3/\text{cm}^2$ to obtain a low density (~ 20-30% confluent) or $2.1 \times 10^4/\text{cm}^2$ to reach a high cell density (~100% confluent) within 48 hours. The phase-contrast images in the top panel of Figure 4A show typical morphology of C2C12 myoblasts at low or high cell densities. The middle panel of Figure 4A shows that the protein abundance of cleaved caspase-3 and Poly (ADP-ribose) polymerase (PARP) was markedly increased in fully confluent cells as compared with cells that were plated at a low density. The cleavage of PARP by caspases leads to nuclear DNA fragmentation. Furthermore, a cell death ELISA assay showed an elevation of cytosolic nucleosomes at full cell confluence (bottom panel, Figure 4A). This provided additional evidence for an increase in apoptotic DNA fragmentation in confluent cells compared with non-confluent cells. We next explored the expression pattern and level of M-cadherin at different cell densities. As shown in the top panel of Figure 4B, M-cadherin was located diffusely throughout the cells that were plated at a low density. In contrast, M-cadherin relocated to cell-cell contacts at

the periphery of the cells when they were confluent. The protein abundance of M-cadherin increased in confluent C2C12 myoblasts compared with the non-confluent cells (bottom panel, Figure 4B). To investigate the role of M-cadherin in regulating myoblast survival and apoptosis, we inhibited M-cadherin expression in confluent C2C12 myoblasts via transient transfection with M-cadherin-targeted small interfering RNA (siRNA). Figure 4C shows that the knockdown of M-cadherin expression in confluent C2C12 myoblasts causes a disruption in the cell-cell contacts and this increased the separation between the cells. Cells in the control group adhered tightly to each other as the cells became confluent (Figure 4C). The knockdown of M-cadherin was verified by immunoblotting (Figure 4D). In control groups, the M-cadherin signals were detected in a multi-band pattern because most cadherins undergo various posttranslational modifications and truncation to get to their mature form (34). The protein abundance of N-cadherin in C2C12 cells was not affected by M-cadherin RNAi treatment (Figure 4D). M-cadherin RNAi exacerbated cell confluence-induced apoptosis in C2C12 cells as determined by the increased protein abundance of cleaved caspase-3 and cleaved PARP (Figure 4D). An increase in DNA fragmentation as measured by a cell death ELISA assay (Figure 4E) confirmed that M-cadherin RNAi increased the level of cell confluence-induced apoptosis.

Apoptosis is initiated in muscle by three pathways. These include the extrinsic death receptor-mediated, the intrinsic mitochondrial-dysfunction associated and the intrinsic endoplasmic reticulum-dysfunction associated pathways (10; 35; 36). To further clarify which apoptotic pathway was most likely involved in cell death caused by M-cadherin

RNAi, we examined the activation status of caspase-9 and caspase-8, which are representative initiation caspases for the intrinsic or the extrinsic apoptotic pathway, respectively. The protein abundance of cleaved caspase-9 but not caspase-8 was markedly increased in response to M-cadherin RNAi (Figure 4F). In addition, the protein abundance of cytosolic cytochrome c and nuclear apoptosis inducing factor (AIF) were also increased upon M-cadherin RNAi. This suggests that the apoptosis that was induced by M-cadherin RNAi was mediated via the intrinsic mitochondria-associated pathway. To further investigate the impact of M-cadherin RNAi on mitochondria as a mediator of apoptosis, we examined the cardiolipin content of the inner mitochondrial membrane of live cells using nonyl acridine orange (NAO) staining. NAO is a metachromatic dye that binds specifically to the mitochondrial cardiolipin and its fluorescence intensity is an indicator of mitochondrial integrity (37; 38). The median fluorescence intensity of NAO staining in M-cadherin-RNAi treated cells was significantly lower than that in control cells (Figure 4G). This indicates that the integrity of mitochondria in C2C12 cells was disrupted after M-cadherin RNAi treatment. Furthermore, the mitochondria membrane potential ($\Delta\psi_{mt}$) was also disrupted by reducing M-cadherin in confluent cells, as shown by a decrease in the orange/green signals from M-cadherin RNAi treated mitochondria compared with the control mitochondria after incubation with the fluorescent probe JC-1 (Figure 4H). JC-1 is a mitochondria permeable lipophilic cation that changes its color from orange to green as the $\Delta\psi_{mt}$ decreases. A reduced $\Delta\psi_{mt}$ leads to a shift of the emitted light from 590nm (orange) to 530nm (green) (39).

M-cadherin RNAi sensitizes C2C12 myoblasts to serum-starvation-induced

apoptosis. To further explore M-cadherin's role in regulating mitochondrial integrity and cell survival/apoptosis during myogenic differentiation, M-cadherin-RNAi-treated or control C2C12 myoblasts were cultured in serum-free medium. Mitochondria were isolated and $\Delta\psi_{mt}$ was assessed by JC-1 staining after 0, 6, 12, 24 or 48 hr of serum starvation. There was a transient decrease in the ratio of JC-1 orange/green staining of isolated mitochondria from all groups of cells in response to serum starvation, but the M-cadherin-RNAi-treated group had the lowest $\Delta\psi_{mt}$ at all time points compared with the control cells (Figure 5A). These results show that knocking down M-cadherin by RNAi reduced $\Delta\psi_{mt}$ during serum starvation. The level of apoptotic DNA fragmentation was significantly increased in M-cadherin RNAi-treated cells that were still attached to the plates at all time points of serum starvation compared with the control groups (Figure 5B). Furthermore, quantification of apoptotic cells from both attached and floating cell populations by TUNEL staining, demonstrated that there was a significant increase in the number of cells undergoing apoptosis during serum starvation in M-cadherin RNAi-treated cells as compared with the control groups (Figure 5C). In addition, there are fewer cells that remained attached to the plates in the M-cadherin RNAi-treated group as compared with control groups when serum starvation progressed to longer time periods (Figure 12). Serum starvation caused an acute activation of cleaved caspase-9 in C2C12 myoblasts, which is indicative of activation of the mitochondrial-associated apoptotic pathway. The protein abundance of cleaved caspase-9 was significantly higher in M-cadherin RNAi treated cells at all time points of serum starvation as compared with control cells (Figure 13). This suggests that

knockdown of M-cadherin expression sensitizes C2C12 myoblasts to serum-starvation-induced apoptosis.

M-cadherin-mediated signaling enhances PI3K/Akt-dependent inhibitory phosphorylation of GSK-3 β ^{ser9}. Akt is a key mediator of survival signaling pathways, and it is associated with cadherin signaling in various cells (40; 41). Since GSK-3 β , another key regulator of cell fate and a phosphorylation target of Akt, is associated with muscle wasting, we also examined the change in GSK-3 β phosphorylation in response to M-cadherin RNAi treatment in confluent cells. As a functional read-out for GSK-3 β activation, the protein abundance of two targets for GSK-3 β , cyclin D1 (42) and survivin (43) were measured. M-cadherin RNAi significantly reduced the activation of Akt in confluent C2C12 cells as determined by the expression of phospho-Akt^{ser473} (Figure 6A). Consequently, the inhibitory phosphorylation of GSK-3 β ^{ser9} was decreased in cells that were treated with M-cadherin RNAi. Quantification of the immunoblot signals from four independent experiments showed a statistically significant and a reproducible (bottom panel of Figure 6A and Figure 15A-D) reduction in phospho-Akt^{ser473}, GSK-3 β phosphorylation, survivin and cyclin D1 in C2C12 cells after M-cadherin RNAi treatment. Conversely, the activation of M-cadherin-mediated signaling via a recombinant M-cadherin-Fc chimera treatment induced a significant increase in phosphorylation of Akt and GSK-3 β at the corresponding residues (Figure 6B and Figure 16A, 16B). The protein abundance of survivin and cyclin D1, were increased in response to recombinant M-cadherin-Fc chimera treatment (Figure 6B; Figure 16C, 16D). Immunoprecipitation data show that the binding between the p85 α subunit of PI3K and

M-cadherin was increased in response to recombinant M-cadherin-Fc chimera treatment (Figure 6C). The protein abundance of PI3K-p85 α in the membrane fraction was markedly increased in M-cadherin-Fc-treated cells compared with the control cells (Figure 6D). Treatment with the PI3K inhibitor, Wortmannin, but not the MEK-1 inhibitor, PD9805, during the last 6 hours of M-cadherin-Fc treatment, completely prevented the increase in phospho-Akt^{ser473} and phospho-GSK-3 β ^{ser9}. This shows that the increased inhibitory phosphorylation of GSK-3 β that is induced by M-cadherin-mediated signaling, is PI3K dependent (Figure 6E).

GSK-3 β overexpression exacerbates, whereas M-cadherin-Fc treatment attenuates apoptosis induced by cell confluence or serum starvation. To further characterize the role of GSK-3 β in regulating apoptosis, C2C12 myoblasts that were 80% confluent were transiently transfected with a wild-type GSK-3 β plasmid and allowed to grow for 48 hours until they reached overconfluence. The transfection efficiency of the GSK-3 β plasmid by FuGENE 6 in C2C12 cells was estimated to be ~20-30% (Figure 14A). Immunoblot analysis confirmed that transfection with GSK-3 β increased the protein abundance of GSK-3 β and decreased the protein level of cyclin D1 (Figure 7A), a target of GSK-3 β phosphorylation that induces its degradation. Overexpression of wild-type GSK-3 β in confluent C2C12 myoblasts increased the protein abundance of cleaved caspase-9, caspase-3, and PARP. This effect was reversed by co-treating the cells with recombinant M-cadherin-Fc (Figure 7B and 7C). In addition, $\Delta\psi_{mt}$ was decreased in mitochondria from C2C12 cells upon overexpression of wild-type GSK-3 β , which was rescued by recombinant M-cadherin-Fc co-treatment

(Figure 7D). Together these data indicate that overexpression of wild-type GSK-3 β alone is sufficient to exacerbate the mitochondria-associated apoptosis induced by cell confluence and this effect can be reversed by enhancement of M-cadherin-mediated signaling via M-cadherin-Fc treatment. Furthermore, in response to serum starvation, the wild-type GSK-3 β plasmid-transfected cells had significantly higher cell death than the control cells at all time points, suggesting that overexpression of wild-type GSK-3 β sensitized the cells to serum starvation-induced apoptosis (Figure 7E). In contrast, co-treatment of C2C12 cells with M-cadherin-Fc and wild-type GSK-3 β overexpression after 48 hours of serum starvation significantly attenuated cell death, as compared to GSK-3 β overexpression alone (Figure 7F).

GSK-3 β inhibition attenuates the apoptosis exacerbated by M-cadherin RNAi. The role of GSK-3 β inhibition on M-cadherin's role in regulating apoptosis was examined using a myristoylated Akt mutant (myrAkt) which functions as a constitutively active form of Akt (44), to inhibit GSK-3 β activity, and a V5-tagged kinase-deficient mutant GSK-3 β (K85R), which functions as a dominant negative GSK-3 β (45). The transfection efficiency of these plasmids using DharmaFECT Duo in C2C12 myoblasts was estimated to be 25-35% as determined from transfecting studies using the pEGFP-C3 vector and DharmaFECT DUO as the transfection agent (Figure 14B).

To evaluate the effects of GSK-3 β inhibition on apoptotic signaling, C2C12 cells that were 80% confluent were transfected with M-cadherin-targeted siRNA, plus either the myrAkt or the GSK-3 β (K85R) plasmid, or an empty vector. The cells were allowed to grow for 48 hours at which time they had reached overconfluency. As expected, there

was a significant increase in both phospho-Akt and total-Akt abundance in the cells transfected with the myrAkt plasmid (Figure 8A, right panel). In addition, there was an increase in total GSK-3 β and cyclin D1 protein abundance in the cells that were transfected with GSK-3 β K85R plasmids (Figure 8A, left panel). Forced expression of myrAkt or GSK-3 β K85R plasmids significantly attenuated apoptosis as seen by lower levels of cleaved-caspase-9, cleaved-caspase-3, and cleaved-PARP (Figure 8B and 8C) after M-cadherin RNAi treatment or overconfluency. Furthermore, co-transfection of myrAkt or GSK-3 β K85R plasmid with M-cadherin-targeted siRNA significantly reduced DNA fragmentation that was caused by 48-hours serum starvation alone or exacerbated by M-cadherin RNAi treatment (Figure 9A). To further confirm the impact of GSK-3 β inhibition on M-cadherin's regulatory effect on apoptosis, we transfected the cells with M-cadherin-targeted siRNA for 36 hours followed by treatment with TDZD-8 (20 μ M) for 12 hours. TDZD-8 is an established non-ATP competitive inhibitor for GSK-3 β (46). TDZD-8 treatment abrogated apoptosis and reversed the loss of $\Delta\psi_{mt}$ that was caused by M-cadherin RNAi following a period of 48-hours of serum starvation (Figure 9B, 9C).

GSK-3 β inhibition partially restores the myogenic differentiation impaired by M-cadherin RNAi. Lastly, we sought to investigate the impact of M-cadherin RNAi and GSK-3 β inhibition on the outcome of myogenic differentiation. M-cadherin-RNAi C2C12 myoblasts treated with or without TDZD-8 were cultured in differentiation medium for 48 hours to induce myogenic differentiation. Immunofluorescent staining of myosin heavy chain (MyHC) was used as a terminal myogenic marker. A TUNEL assay was performed to determine the level of *in situ* apoptotic DNA fragmentation. Knockdown of

M-cadherin by RNAi significantly impaired myogenic differentiation, as many cells died and detached from the plates. In contrast, TDZD-8 treatment partially restored the myogenic differentiation that was blocked by M-cadherin RNAi. This was determined by the myoblast fusion index, which represented the percentage of myoblasts that matured into myotubes (Figure 10A, 10B). Calculation of the apoptotic index from TUNEL labeling showed that TDZD-8 treatment significantly attenuated the number of apoptotic nuclei that was caused by M-cadherin RNAi over 48-hours of myogenic differentiation (Figure 10A, 10C).

These findings were further verified in mouse primary myoblasts. Syndecan-4-positive primary myoblasts were isolated by fluorescence activated cell sorting (FACS) as representative muscle progenitor cells (18; 47). ~20% of the adult muscle stem cells were Syndecan-4 positive (Figure 11A). Knockdown of M-cadherin by RNAi significantly impaired myotube formation from Syndecan-4-positive primary myoblasts, which was partially rescued by TDZD-8 treatment as shown from MyHC staining and a greater fusion index (Figure 11B and 11C) in the TDZD-8 treated cells. Furthermore, M-cadherin RNAi exacerbated apoptosis in Syndecan-4-positive primary myoblasts after 48-hours of myogenic differentiation. Apoptosis could be abrogated by TDZD-8 treatment, as shown by a lower TUNEL staining (Figure 11B) and an apoptotic index (Figure 11D) after TDZD-8 treatment.

DISCUSSION

This is the first report to show that M-cadherin-mediated signaling protects myoblasts against apoptosis during myogenic differentiation. *In vitro* culture of myoblasts at high cell density has been shown to yield not only a better myogenic differentiation outcome, but also an increased incidence of apoptosis, as compared to culturing the cells at a low density (4). This observation is consistent with the suggestion that apoptosis and differentiation are tightly regulated in a coordinate pattern in myoblasts (3). In the current study we show that M-cadherin protein abundance is increased and M-cadherin engagement at cell-cell contacts is induced when myoblasts become confluent. While confluence triggers apoptosis during differentiation, other cells survive, differentiate and fuse into myotubes (3; 4; 48-50).

In this study we show that the disruption of M-cadherin signaling by M-cadherin RNAi, sensitized C2C12 myoblasts to apoptosis that was induced by either cell confluence or serum starvation. Apoptosis in C2C12 cells is accompanied by decreased $\Delta\psi_{mt}$, increased mitochondrial release of cytochrome *c* and AIF, and consequently an increased cleavage of caspase-9 but not caspase-8. Together these data indicate M-cadherin RNAi-induced apoptosis is mediated by the intrinsic mitochondria-associated pathway and M-cadherin-mediated signaling plays an important role in maintaining the mitochondrial integrity of differentiating myoblasts and suppressing apoptosis during myogenic differentiation. Our findings are consistent with data showing that $\Delta\psi_{mt}$ is compromised in myoblasts undergoing apoptosis but not those that successfully

differentiate (51). Furthermore, proper mitochondria function is critical for successful myogenic differentiation (37; 52; 53).

Previous findings have shown that in aged muscle, the number of M-cadherin-positive satellite cells is decreased (25) but the apoptotic propensity of satellite cells is increased compared with those in young animals (20; 21). This suggests that there may be a negative relationship between the expression level of M-cadherin and the apoptosis susceptibility of muscle progenitor cells. In the current study, we show that M-cadherin has a protective role against apoptosis during myogenic differentiation since inhibition of M-cadherin expression by RNAi, sensitized both C2C12 and Syndecan-4-positive primary myoblasts to apoptosis, which resulted in an increase in pro-apoptotic markers, and resulted in an impaired myotube formation as compared to control cells. We speculate that reduced M-cadherin might be a contributing mechanism that would explain, at least in part, an increased susceptibility of muscle progenitor cells to increased apoptotic signaling and elimination of nuclei in aged muscles (10; 20; 21; 36).

GSK-3 β is a serine/threonine kinase that is constitutively active in resting cells and plays a key role in regulating glucose homeostasis (54). GSK-3 β is also important in regulating apoptosis. However, it has a paradoxical effect on apoptosis from different pathways by promoting mitochondrial-dysfunction-associated apoptosis signaling, but also inhibiting death-receptor-mediated apoptosis signaling (55). Dysregulation of GSK-3 β has been reported to be involved in several pathologies including neurodegenerative diseases, mood disorders, cancer, and diabetes (55). Studies in skeletal muscles have

linked GSK-3 β to muscle wasting conditions. GSK-3 β activity has been shown to increase in aged (56) and burn-injured skeletal muscles (57). Inhibition of GSK-3 β is required for IGF-1-induced myogenic differentiation (58). Furthermore, GSK-3 β inhibition reduces protein degradation in conditions of muscle wasting and may also promote re-growth of atrophic muscle (59-61). Although a conditional skeletal muscle knockout of GSK-3 β has been reported to manifest a phenotype with an improved insulin tolerance and glucose metabolism in muscle tissues (62), currently, there are no data that evaluate a direct role of GSK-3 β in regulating the muscle progenitor cell survival and apoptosis during myogenic differentiation. In this study we show for the first time that overexpression of wild-type GSK-3 β is sufficient to induce apoptosis in confluent C2C12 myoblasts and to sensitize the cells to serum-starvation-induced apoptosis. This effect can be attenuated by increasing M-cadherin-mediated signaling via recombinant M-cadherin-Fc treatment. We also show that the activation of M-cadherin-mediated signaling recruits PI3K and activates Akt, which in turn phosphorylates GSK-3 β at the serine 9 residue, resulting in its suppression. Conversely, inhibition of GSK-3 β activation attenuates apoptosis that is induced by knockdown of M-cadherin expression. This suggests that GSK-3 β plays a critical role in mediating M-cadherin's protective effect against apoptosis during myogenic differentiation. Our findings are consistent with data from Robakis and colleagues (63) who showed that the expression of presenilin-1 in fibroblasts promotes the association of PI3K with E- and N-cadherins and activates Akt, leading to the inhibition of GSK-3 β activity. Skeletal muscle is the largest consumer of glucose and GSK-3 β plays an important role in regulating glucose metabolism and insulin sensitivity in muscle (62; 64). Thus we speculate that

manipulation of M-cadherin-mediated cell-cell adhesion may have a profound impact on glucose metabolism and insulin tolerance of muscle tissue through regulating the activation status of GSK-3 β . Interestingly, a type 2 diabetic phenotypic KK/Ta mouse, has been shown to have an ectopic expression of M-cadherin in liver with three missense mutations (65). Further statistical analysis revealed a correlation between M-cadherin expression and the hypertriglyceridemia, glucose intolerance and hyperinsulinemia in the KK/Ta mouse. This suggests that disrupted M-cadherin signaling may have a role in the pathology of type 2 diabetes (65).

GSK-3 β may regulate mitochondrial outer membrane permeabilization by targeting multiple substrates. The Bcl-2 family of proteins represents one target of GSK-3 β . GSK-3 β directly phosphorylates the pro-apoptotic Bax protein at serine 163, leading to its activation (66). GSK-3 β activation also induces the expression of the pro-apoptotic Bim protein (67). On the other hand, GSK-3 β phosphorylation of MCL-1, an anti-apoptotic member of Bcl-2 family protein, facilitates its degradation (68). Another class of GSK-3 β substrates in regulating apoptosis is a group of transcription factors that include p53, β -catenin, and Myc. p53 plays a crucial role in regulating cell cycle arrest, senescence and apoptosis. GSK-3 β can form a complex with nuclear p53 and this promotes p53-induced apoptosis (69). β -catenin is a key mediator for the canonical Wnt signaling pathway, which plays an important role in promoting cell growth and survival. β -catenin also connects cadherins to the actin cytoskeleton. Together with adenomatous polyposis coli (APC) and axin, GSK-3 β forms the “destruction complex” that phosphorylates β -catenin and promotes its ubiquitination and degradation (70).

Cadherins control both the turnover rate and the subcellular distribution of β -catenin. As a result, β -catenin could be the nexus for the convergence of cadherin and GSK-3 β -mediated signaling in skeletal muscle. Additional studies are required to identify the important upstream mediators and downstream targets for M-cadherin/Akt/GSK-3 β signaling that are responsible for maintaining mitochondrial integrity and suppressing apoptosis in myoblasts; however, this is beyond the scope of the current investigation.

In conclusion, the data in the current study show that M-cadherin plays a critical role in regulating survival versus death via apoptosis of myoblasts during myogenic differentiation *in vitro*. M-cadherin-mediated signaling maintains the inhibitory tone from PI3K/Akt upon GSK-3 β by activating PI3K/Akt. M-cadherin signaling appears to help maintain a balance between apoptosis and differentiation and prevents the acceleration of mitochondrial-associated apoptotic signaling in muscle cells.

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Figure 4

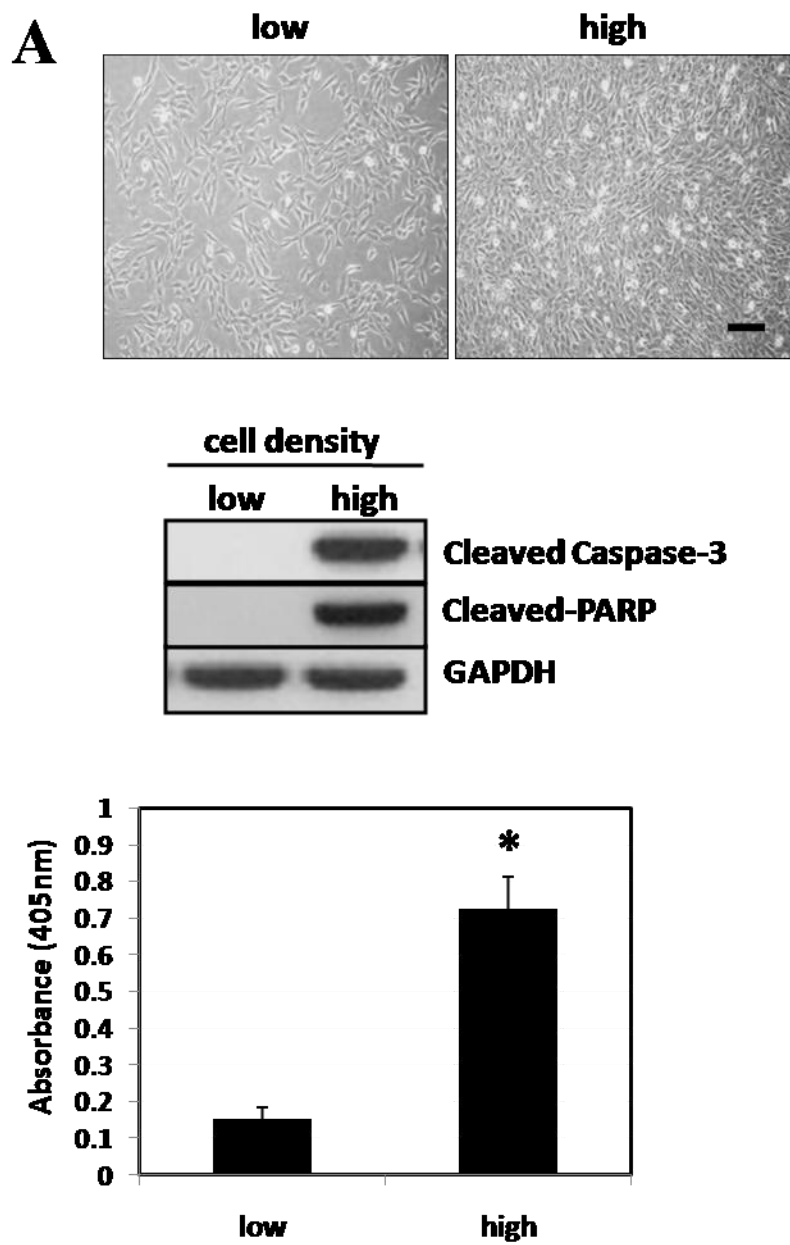


Figure 4

B

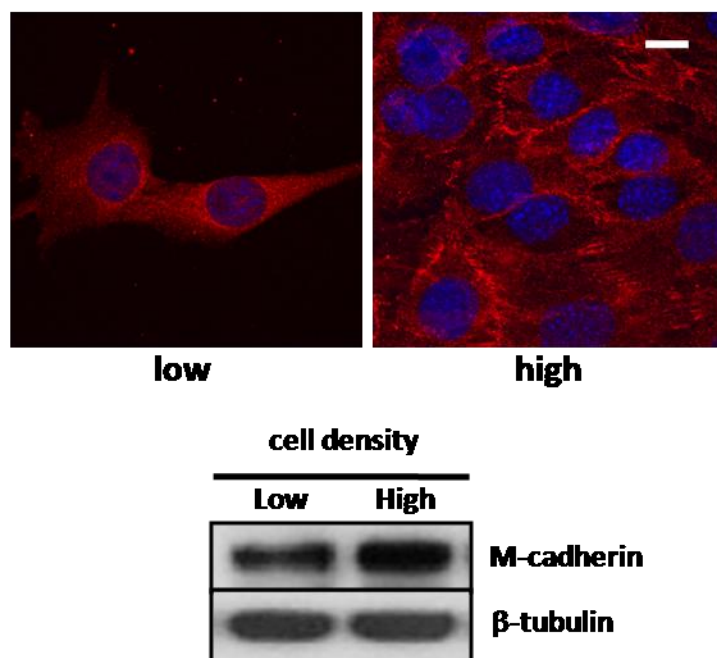


Figure 4

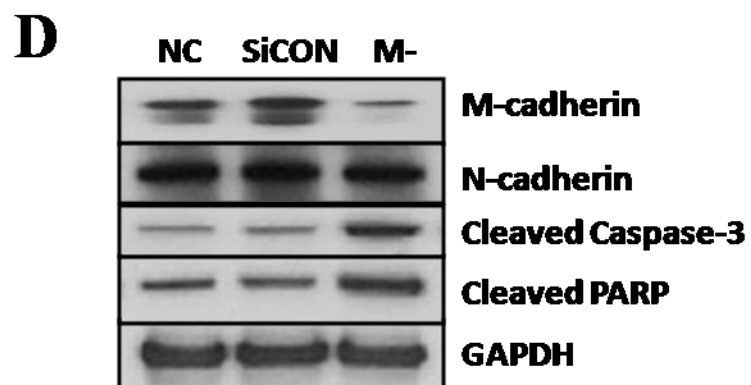
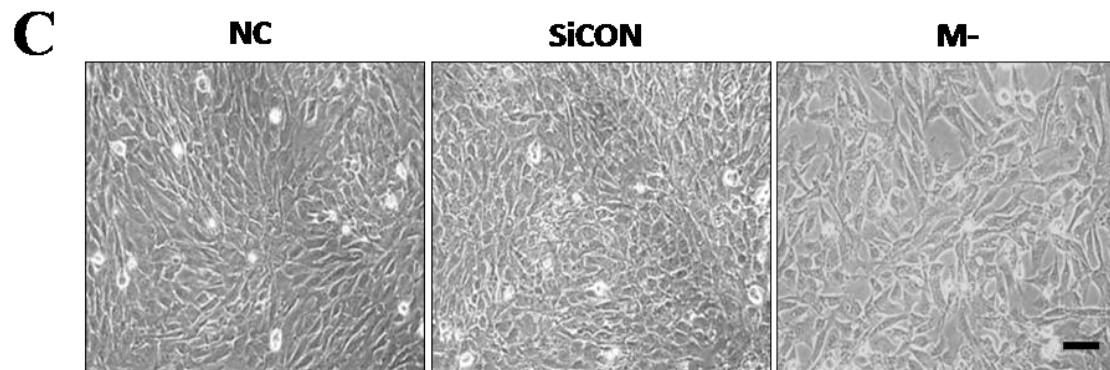


Figure 4

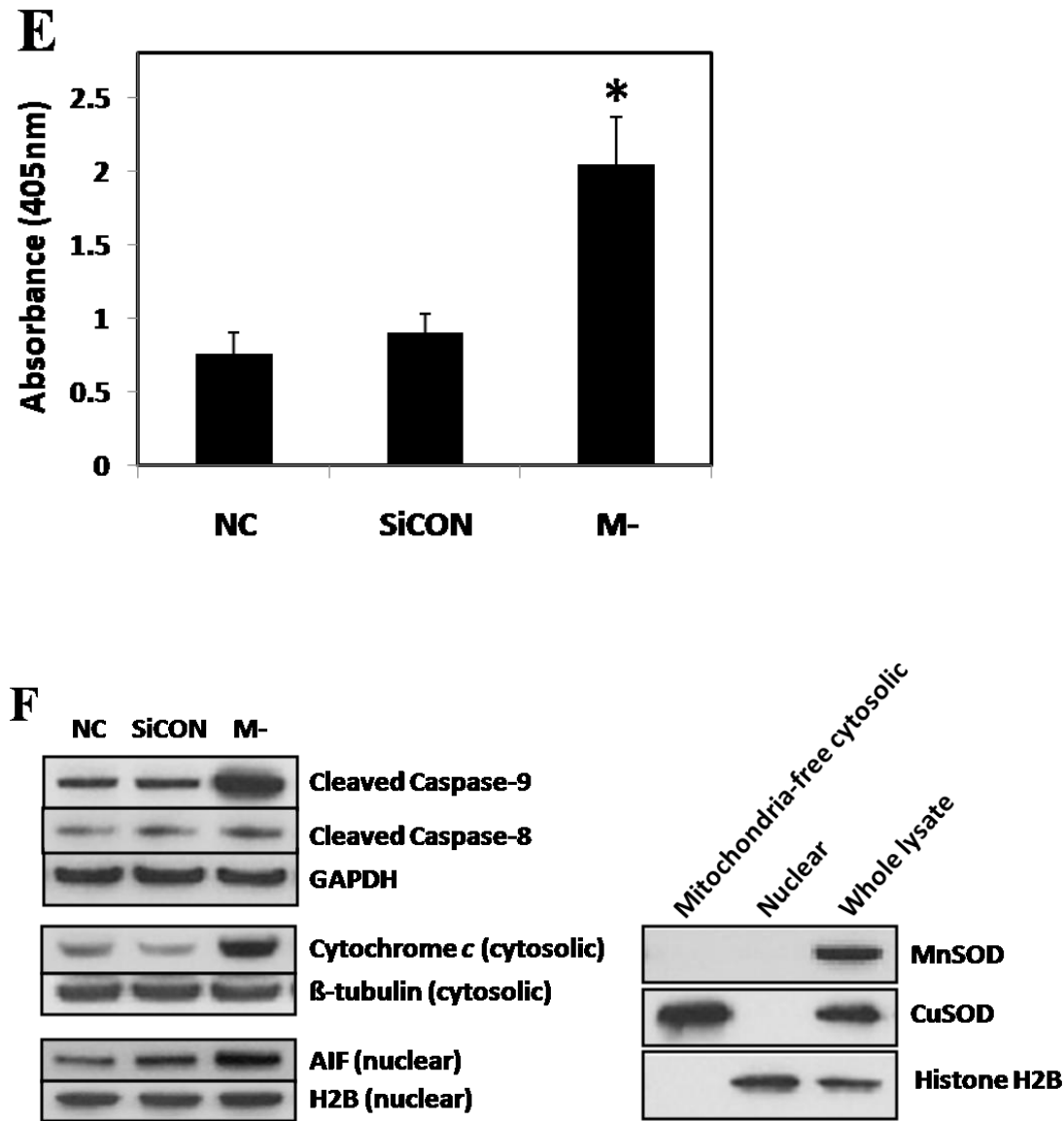


Figure 4

G

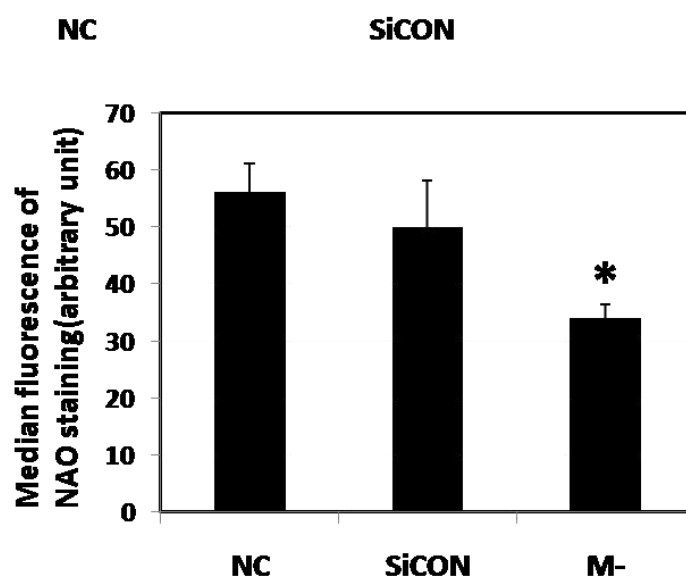
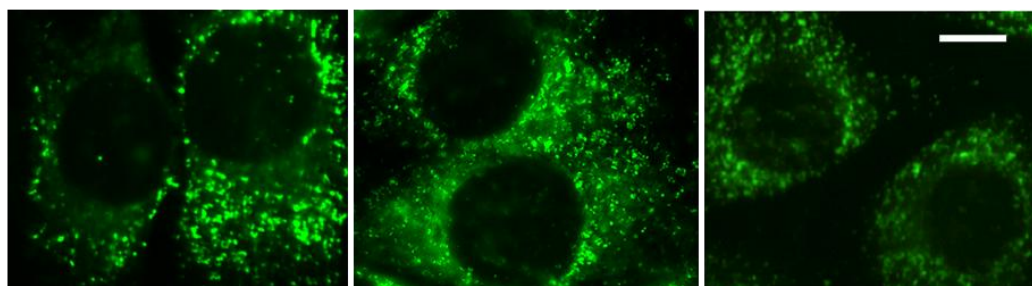


Figure 4

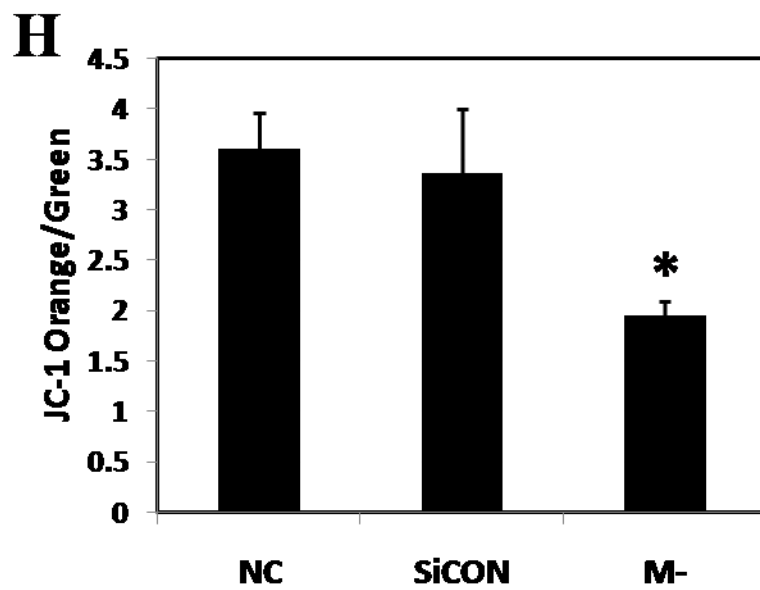


Figure 4. Effect of M-cadherin RNAi on apoptosis in confluent C2C12 myoblasts.

A. Top panel: Representative phase-contrast images of C2C12 myoblasts that were obtained 48 hours after seeding them at a low density ($2.0 \times 10^3/\text{cm}^2$), or a high density ($2.1 \times 10^4/\text{cm}^2$). The objective magnification = 10x. The scale bar = 200 μm . **Middle panel:** Immunoblots of cleaved caspase-3 and PARP obtained 48 hr post plating. **Bottom panel:** Cytosolic nucleosomes of low or high-density cells were measured as an indication of apoptotic DNA fragmentation. Each data point represents the mean \pm SEM of the mean of three independent experiments. *, $P < 0.05$ vs. the low-density group.

B. The expression pattern and protein abundance of M-cadherin at low and high cell densities. **Top panel:** Representative confocal images of M-cadherin (red) and DAPI (blue) staining in C2C12 myoblasts at low or high cell density as described in Figure 1A. The objective magnification = 63x. The scale bar = 10 μm . **Bottom panel:** Immunoblotting analysis of protein abundance of M-cadherin. β -tubulin was probed as a loading control.

C-H, 80% confluent C2C12 myoblasts were transfected with M-cadherin-targeted siRNA (M-) or non-targeted scramble siRNA (SiCON) as a control. Non-transfected cells with identical culture conditions were used as a normal control (NC) cells. The cells were harvested 48hr post-transfection. Each data point (mean \pm SEM) is the mean of three independent experiments. *, $P < 0.05$ vs. the control groups.

C. Phase-contrast images of transfected cells that were acquired at an objective magnification = 20x. The scale bar = 100.

D. Immunoblot analysis of transfected cells.

E. The apoptotic DNA fragmentation of transfected cells is shown as mean \pm SEM of three independent experiments. *, $P<0.05$ vs. both NC and SiCON control groups.

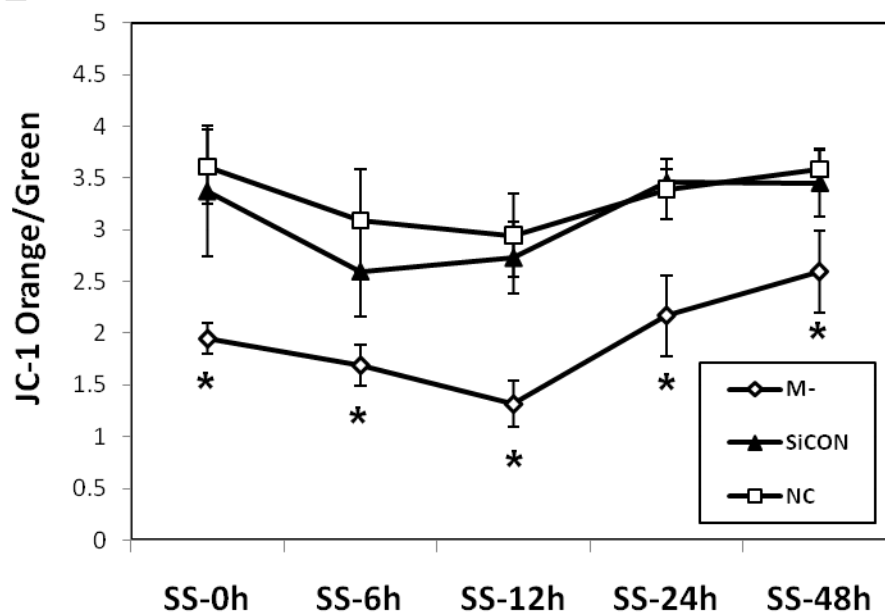
F. Left panel: Proteins associated with apoptotic signaling (and the relevant control proteins) were measured in transfected and control cells. Right panel: The integrity and purity of protein subcellular fractions was verified by immunoblotting with appropriate control antibodies.

G. TOP panel: Digital images were obtained (objective=100x; scale bar = 20 μ m) from control or transfected C2C12 cells that were stained with NAO. Bottom panel: The median fluorescence intensity of NAO per cell was determined. The data are mean \pm SEM of three experiments.

H. Mitochondria were isolated from control and transfected cells and stained with JC-1. The data represent FACS analyses of the ratio of orange (intact mitochondria) to green (compromised $\Delta\psi_m$) mitochondria.

Figure 5

A



B

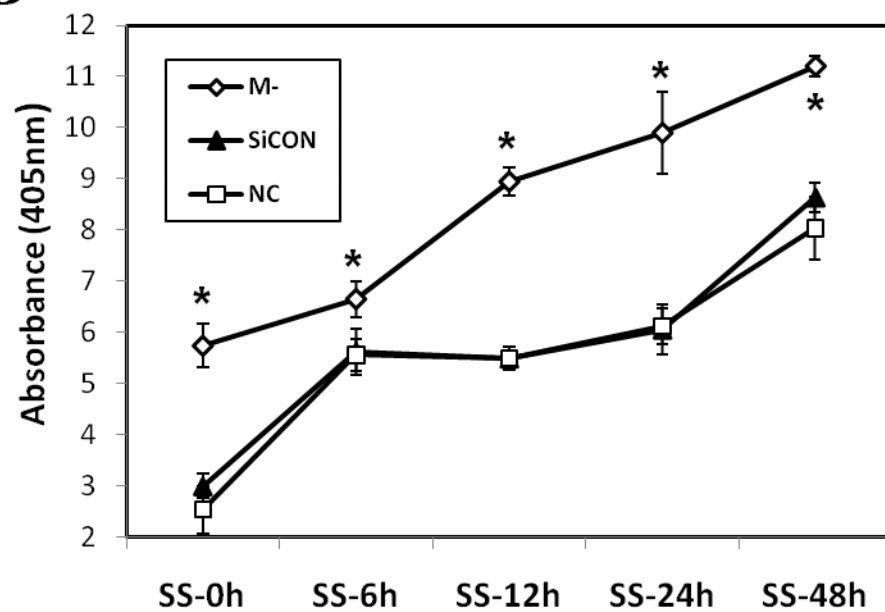


Figure 5

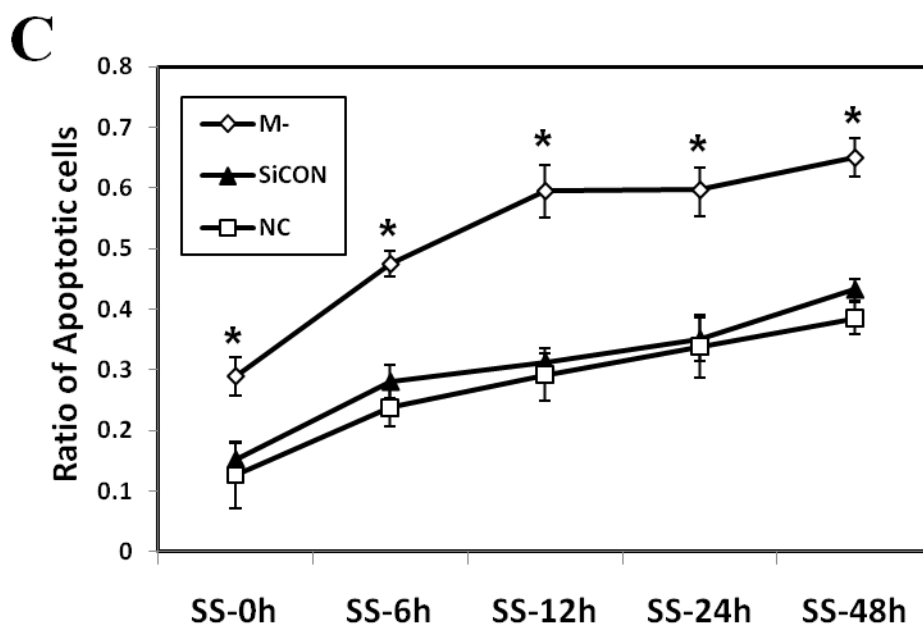


Figure 5. Effect of M-cadherin RNAi on serum starvation-induced apoptosis. M-cadherin-RNAi (M-), non-targeted scrambled siRNA-transfected (SiCON) or normal control (NC) C2C12 myoblasts were serum starved from zero to 48 hours before being harvested. *, $P < 0.05$ vs. both SiCON and NC control groups. The data are shown as the mean \pm SEM for three independent experiments.

A. Mitochondria were isolated from attached cells after serum starvation for 0 hours (SS-0h), 6 hours (SS-6h), 12 hours (SS-12h), 24 hours (SS-24h) or 48 hours (SS-48h). The mitochondria $\Delta\psi_m$ (orange/green ratio) was evaluated by FACS analysis JC-1 staining.

B. DNA fragmentation in attached cells was measured by a cell death ELISA at the same time points as described in A.

C. The percentage of cells undergoing apoptosis in both attached and floating cells were defined as: total TUNEL-positive attached cells + total TUNEL-positive floating cells) divided by (total attached cells + total floating cells).

Figure 6

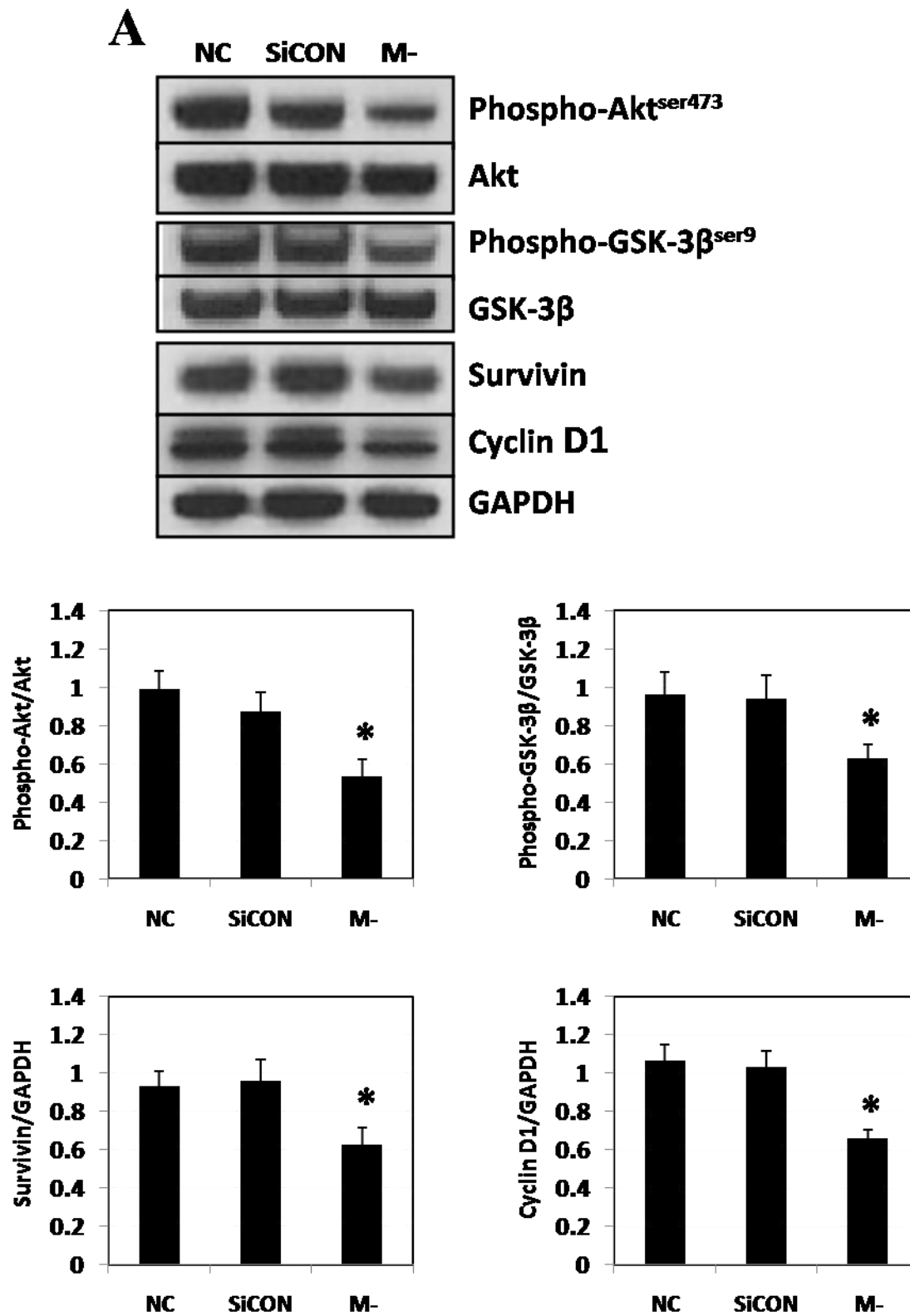


Figure 6

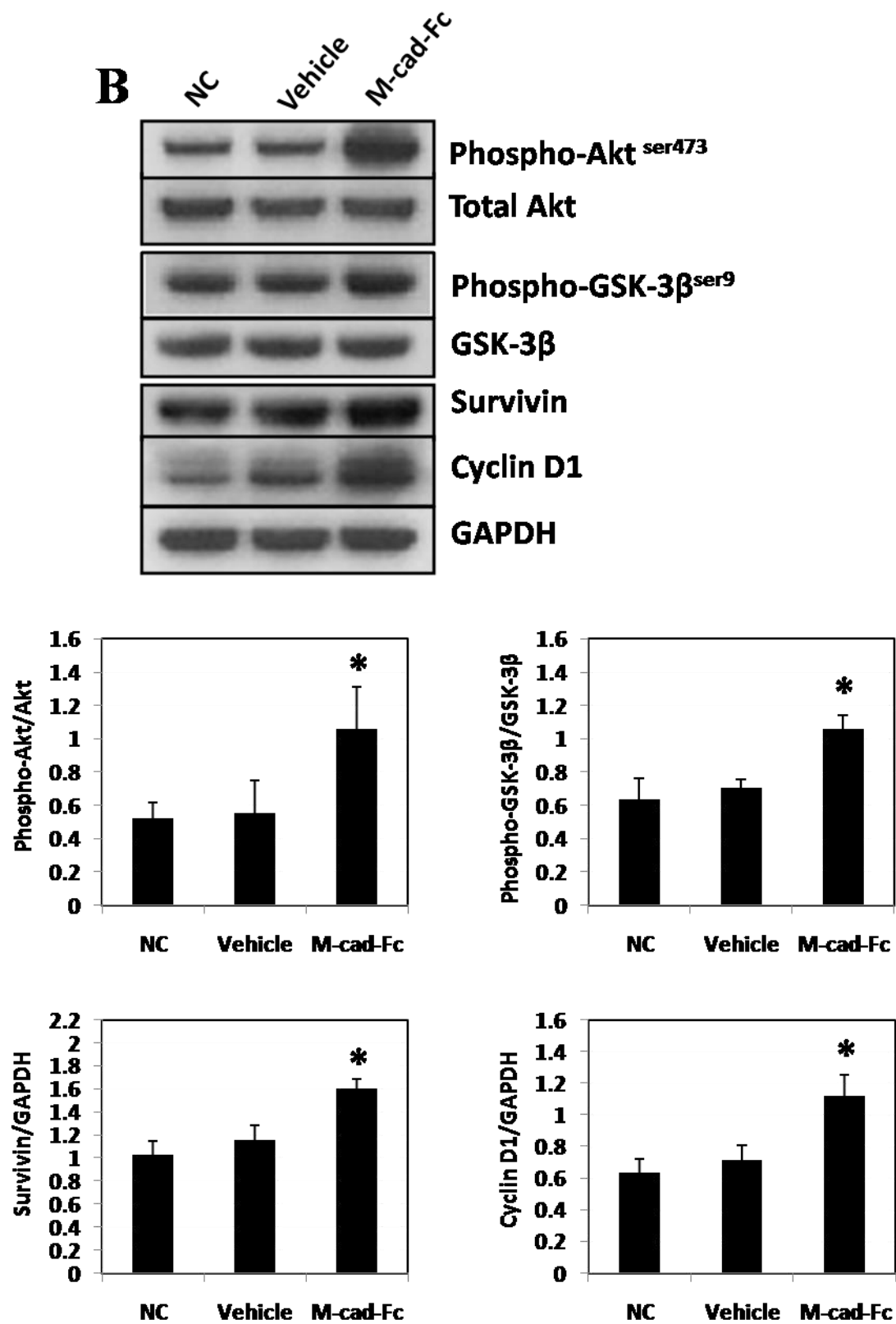


Figure 6

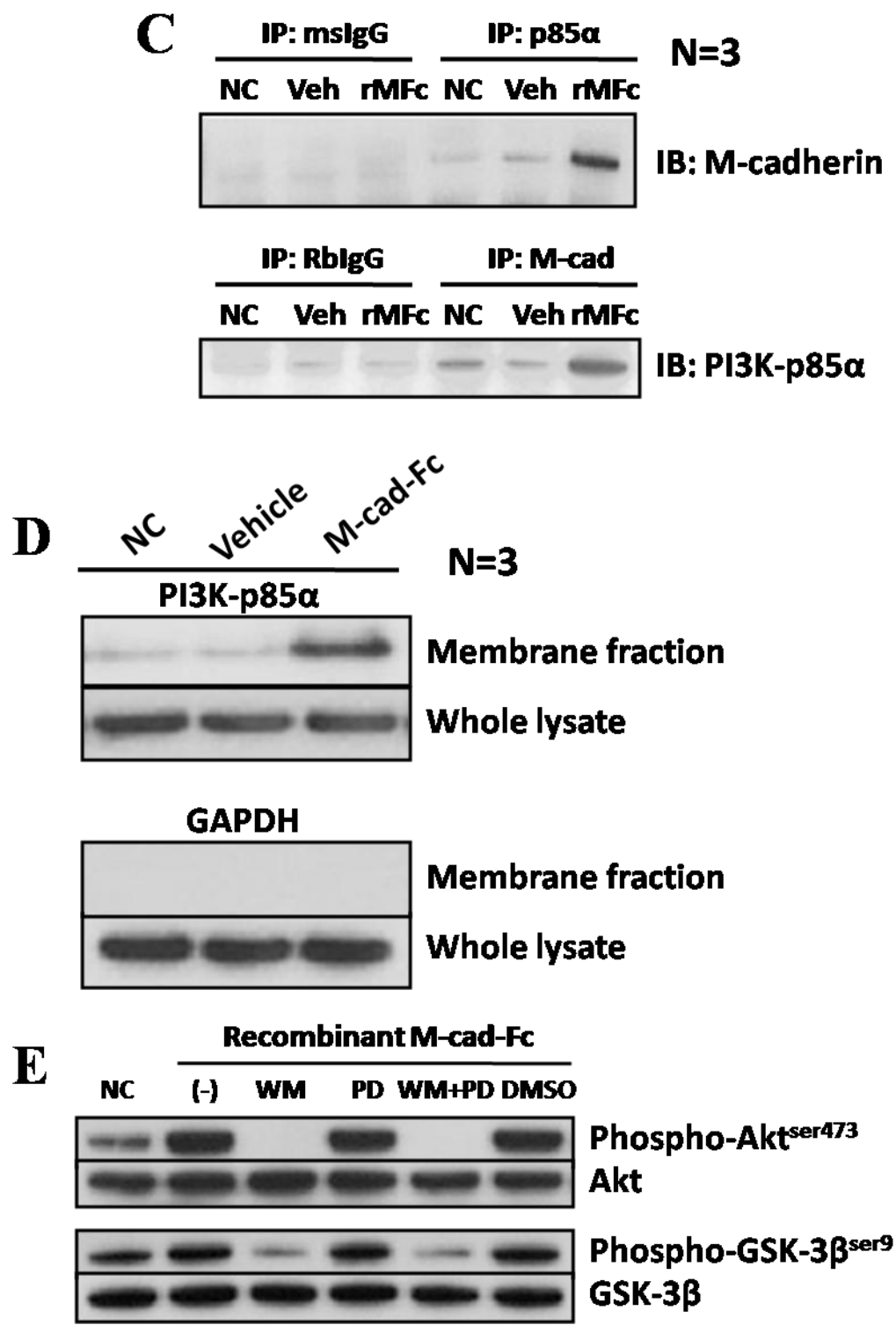


Figure 6. M-cadherin-mediated signaling effect on the PI3K/Akt/GSK-3 β pathway.

A-E. The data are shown as the mean \pm SEM for three independent experiments.

A-B *, $P < 0.05$ vs. the control groups.

A. Top panel: Representative immunoblots of M-cadherin-RNAi (M-) treated, non-targeted scrambled siRNA-transfected (SiCON) treated or normal control (NC) C2C12 cells. Survivin and cyclin D1 were measured as a functional read-out for GSK-3 β activation. GAPDH was used as a loading control. **Bottom Panel:** Densitometric analyses of Akt, GSK-3 β , survivin and cyclin D1 normalized to their loading controls.

B. Top panel: C2C12 myoblasts were grown in normal control (NC), vehicle control (Vehicle) or recombinant M-cadherin-Fc-coated (M-cad-Fc) dishes for 48 hours. The cells were lysed and subjected to immunoblotting analysis of the same proteins as measured in Fig. 3A. **Bottom Panel:** Densitometric analyses of immunoblot band intensities of phosphorylated and total Akt and GSK-3 β , as well as survivin and cyclin D1 in response to recombinant M-cadherin-Fc treatment. The band intensities of the above proteins were normalized by corresponding control proteins.

C. Immunoprecipitation assays of untreated C2C12 myoblasts (NC) or myoblasts treated with either the vehicle (Veh) or with recombinant M-cadherin-Fc (rMFC) were conducted 48 hr after treatment with either a mouse anti-PI3K-p85 α or a rabbit anti-M-cadherin antibody respectively. Western blots of the protein abundance of M-cadherin or PI3K-p85 α were conducted in the precipitated proteins.

D. Cell membrane fractions were prepared from cells that had been treated as described in Fig. 3C and blotted to detect the protein abundance of PI3K-p85 α .

E. C2C12 myoblasts were treated as described in Fig. 3C. The cells were then treated for a subsequent 12 hours with no vehicle added (-), Wortmannin (WM, 200nM), PD98059 (PD, 50μM), Wortmannin plus PD98059 (WM+PD), or DMSO. Untreated normal control cells (NC) did not receive either the recombinant M-cadherin-Fc or the vehicle treatments. The cells were harvested for immunoblotting analysis.

Figure 7

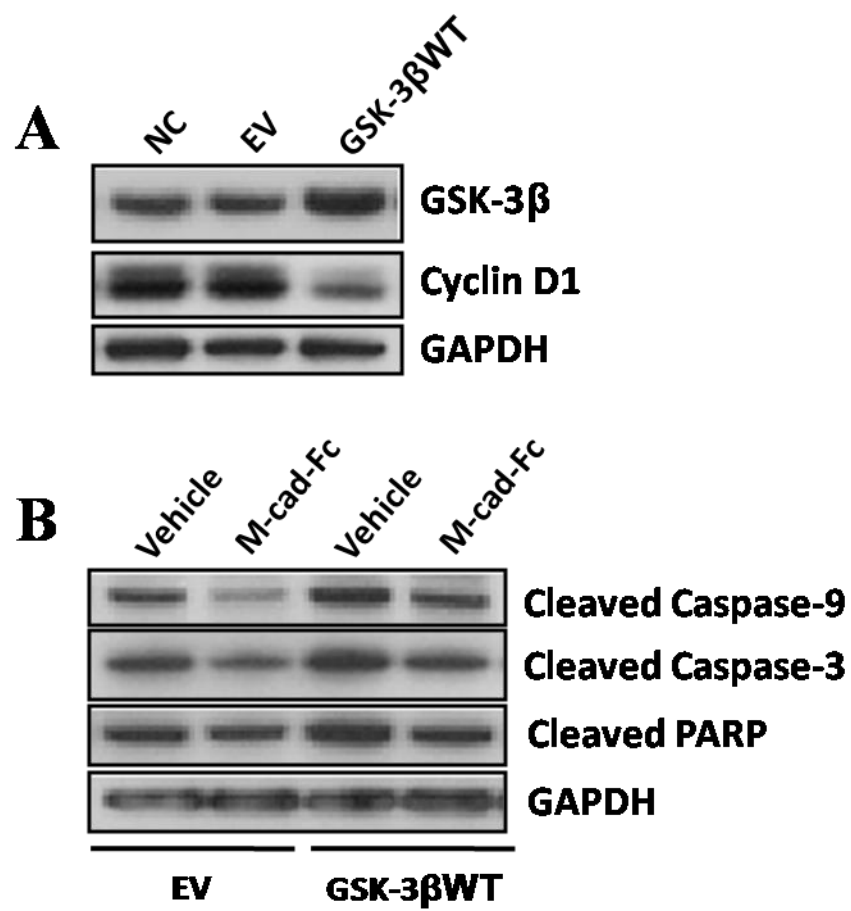


Figure 7

C

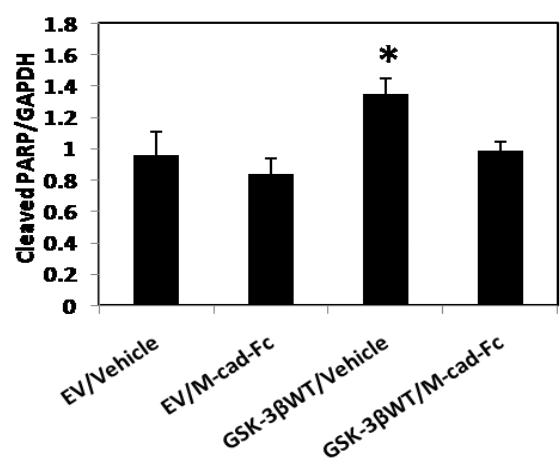
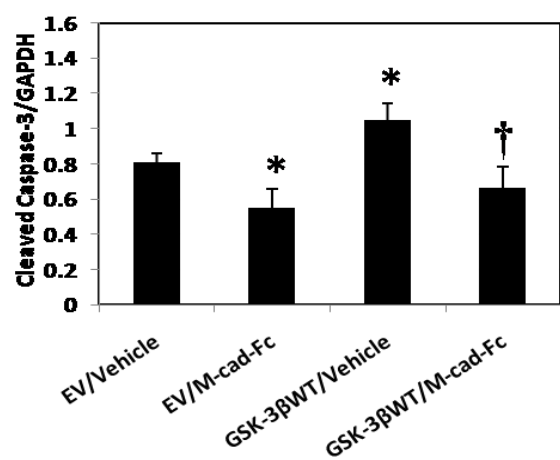
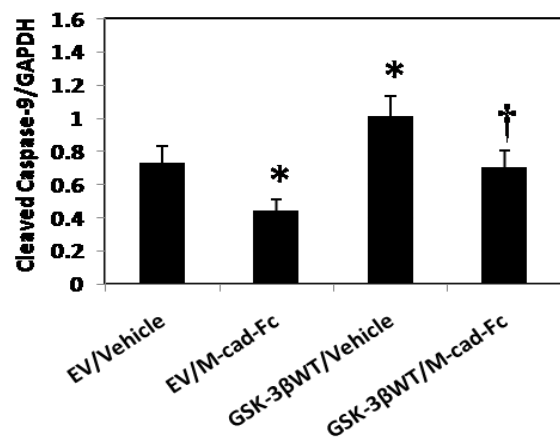
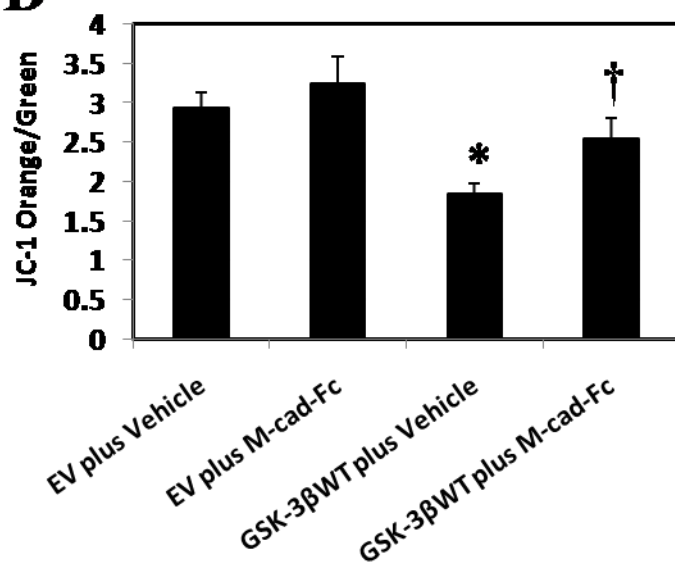


Figure 7

D



E

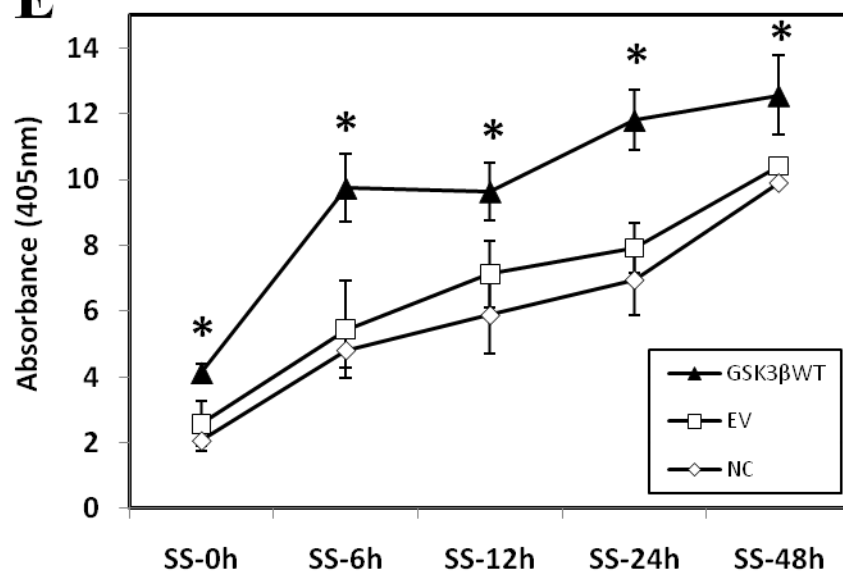


Figure 7

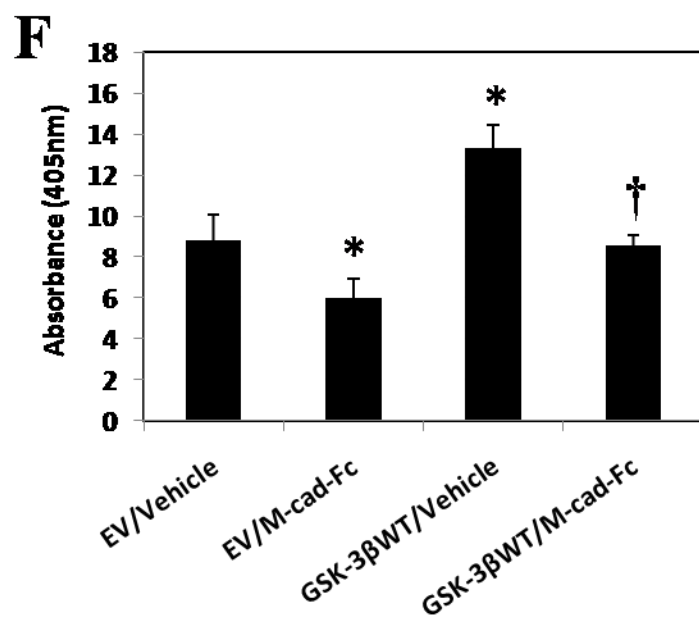


Figure 7. Effect of wild-type GSK-3 β overexpression and recombinant M-cadherin-Fc treatment on apoptosis that was induced by cell confluence or serum starvation.

A. A wild-type GSK-3 β plasmid (GSK-3 β WT) or an empty vector (EV) were transiently transfected into C2C12 myoblasts that were 80% confluent. 48 hours after transfection, the cells were harvested in RIPA buffer and processed for immunoblotting for GSK-3 β and cyclin D1 protein abundance. The experiment was repeated three times under each experimental condition.

B. Wild-type GSK-3 β plasmids (GSK-3 β WT) or empty vectors (EV) were transiently transfected into C2C12 myoblasts growing in recombinant M-cadherin-Fc-coated (M-cad-Fc) or vehicle-coated (Vehicle) dishes. 48 hours later, the cells were harvested in RIPA buffer and immunoblotted against pro-apoptotic proteins. GAPDH was probed as a loading control.

C. Analyses of immunoblot bands described in 4B. The data were normalized to GAPDH and expressed as the mean \pm SEM from three independent experiments. *, $P < 0.05$ vs. EV/Vehicle or EV/M-cad-Fc; †, $P < 0.05$ vs. GSK-3 β WT/Vehicle.

D. Mitochondria were isolated from C2C12 myoblasts that had undergone the same treatments as described in Fig. 4B. The mitochondria were stained with JC-1. A FACSCalibur system was used to measure the change in mitochondrial membrane potential ($\Delta\psi_m$). The data are expressed as the ratio of orange/green JC-1 staining. The data are reported as the mean \pm SEM from three independent experiments. *, $P < 0.05$ vs. EV/Vehicle or EV/M-cad-Fc; †, $P < 0.05$ vs. GSK-3 β WT/Vehicle.

E. C2C12 myoblasts transfected with the wild-type-GSK-3 β plasmid (GSK3 β WT) or the empty vector (EV) and the non-transfected normal control (NC) cells were serum-starved for 0 hours (SS-0h), 6 hours (SS-6h), 12 hours (SS-12h), 24 hours (SS-24h) or 48 hours (SS-48h). At the end of each time point, the attached cells were harvested and DNA fragmentation was measured by a cell death ELISA assay. The data represent the mean \pm SEM from three independent experiments. *, $P < 0.05$, vs. NC or EV.

F. The wild-type GSK-3 β plasmid (GSK3 β WT) or the empty vector (EV) was transiently transfected into C2C12 myoblasts growing in recombinant M-cadherin-Fc-coated (M-cad-Fc) or vehicle-coated (Vehicle) dishes. 48 hours later, the cells were treated with serum starvation for another 48 hours before being harvested. A cell death ELISA assay was used to measure the DNA fragmentation in the harvested cells. The data represent the mean \pm SEM from three independent experiments. *, $P < 0.05$ vs. EV/Vehicle or EV/M-cad-Fc; †, $P < 0.05$ vs. GSK-3 β WT/Vehicle.

Figure 8

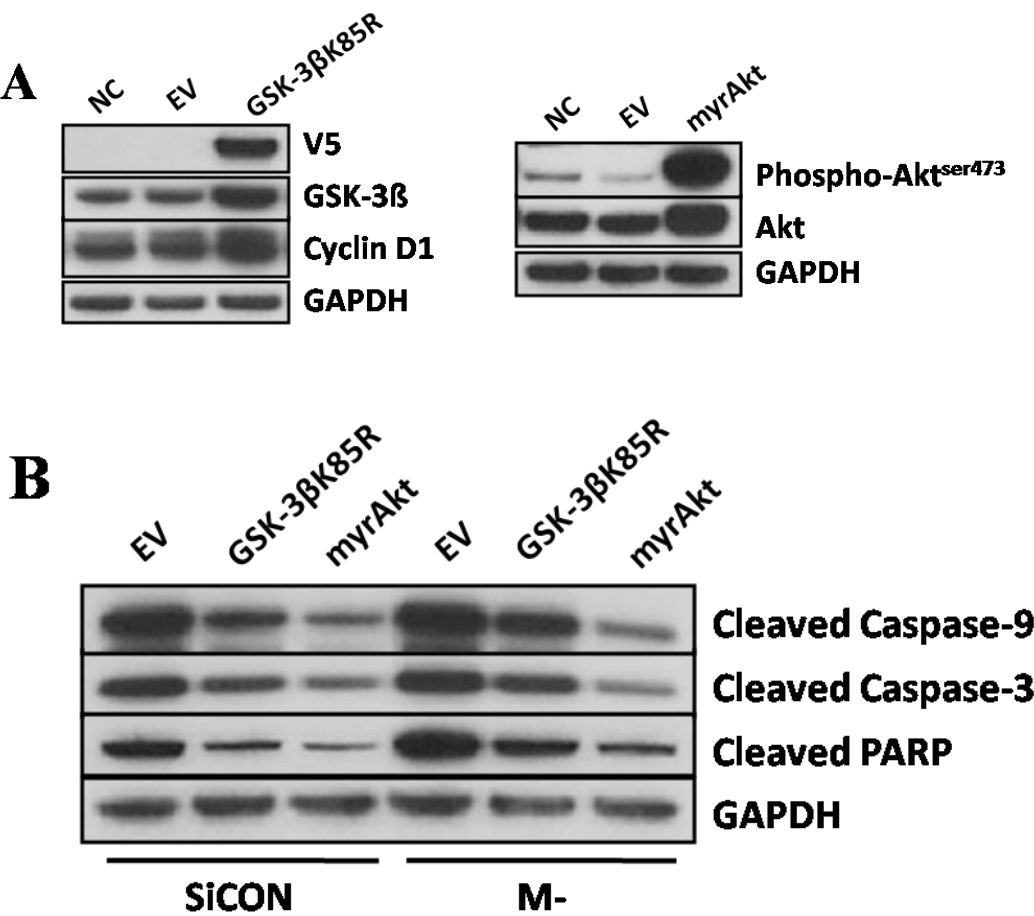


Figure 8

C

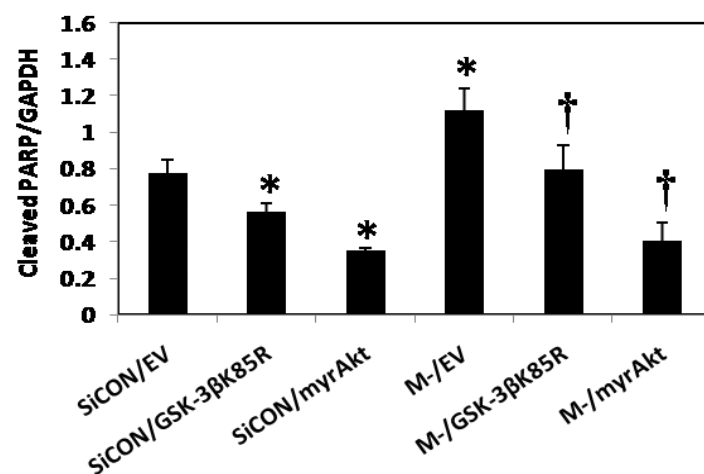
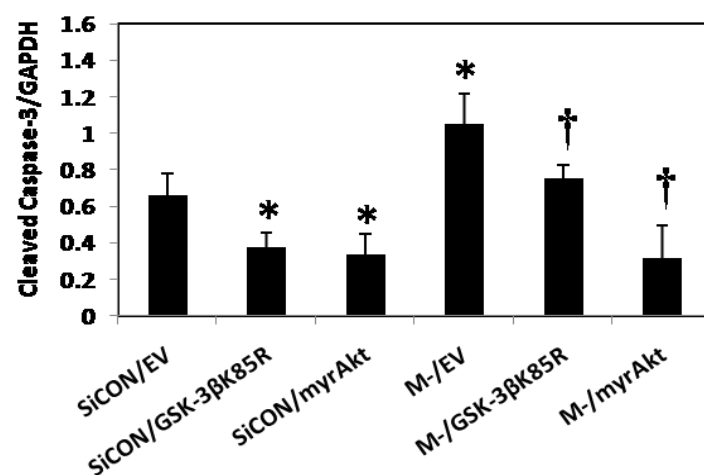
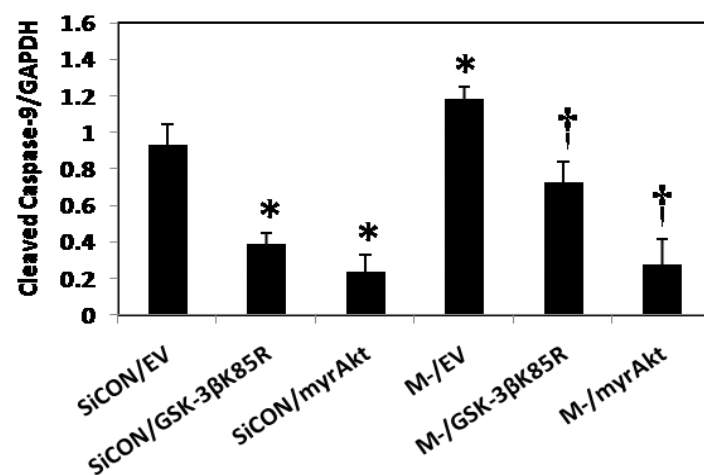


Figure 8. Effect of GSK-3 β inhibition on cell-confluence-induced apoptosis.

A. C2C12 myoblasts were grown to 80% confluency then transfected with an empty vector (EV) or GSK-3 β K85R, which contained a V5 tag (left panel), or constitutively active Akt (myrAkt), (right panel) for 48h. Non-transfected cells normal control cells (NC) were cultured under identical conditions. The protein abundances of V5 tag, GSK-3 β , and cyclinD1 were examined in GSK-3 β K85R plasmid-transfected cells (left panel) and those of serine-437-phosphorylated and total Akt were examined in myrAkt plasmid-transfected cells (right panel) by immunoblotting.

B. C2C12 myoblasts were grown to 80% confluent then co-transfected with the M-cadherin-targeted-siRNA (M-) plus myrAkt (M-/ myrAkt) or the GSK-3 β K85R plasmid (M-/ GSK-3 β K85R) or an empty vector (M-/EV). Similar co-transfections were completed with the non-targeted scramble siRNA (SiCON) with myrAkt (SiCON/ myrAkt), GSK-3 β K85R (SiCON/ GSK-3 β K85R) or the empty vector (SiCON/EV). Forty-eight hours after transfection, the cells were harvested and processed for immunoblotting of cleaved caspase-9, cleaved caspase-3, and cleaved PARP. GAPDH was used as a loading control. Each experiment was repeated three times.

C. Densitometric analyses of immunoblots were obtained from C2C12 cells with identical treatments as described in Figure 5B. The data represent the mean \pm SEM from three independent experiments. *, $P < 0.05$, vs. SiCON/EV; †, $P < 0.05$, vs. M-/EV.

Figure 9

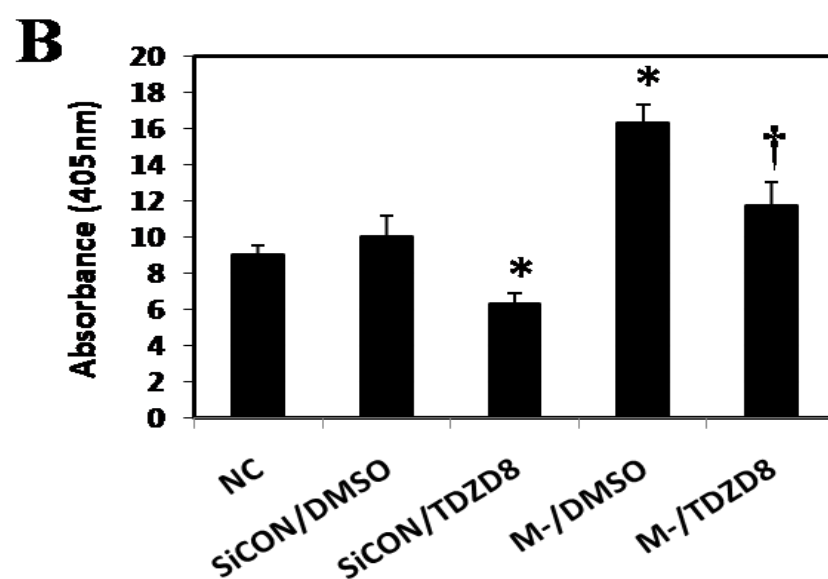
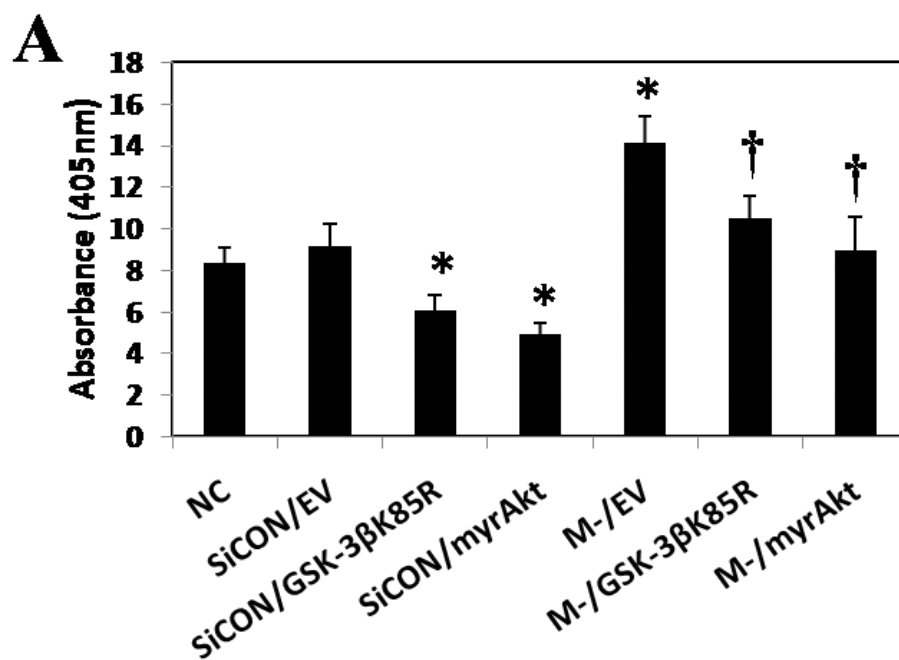


Figure 9

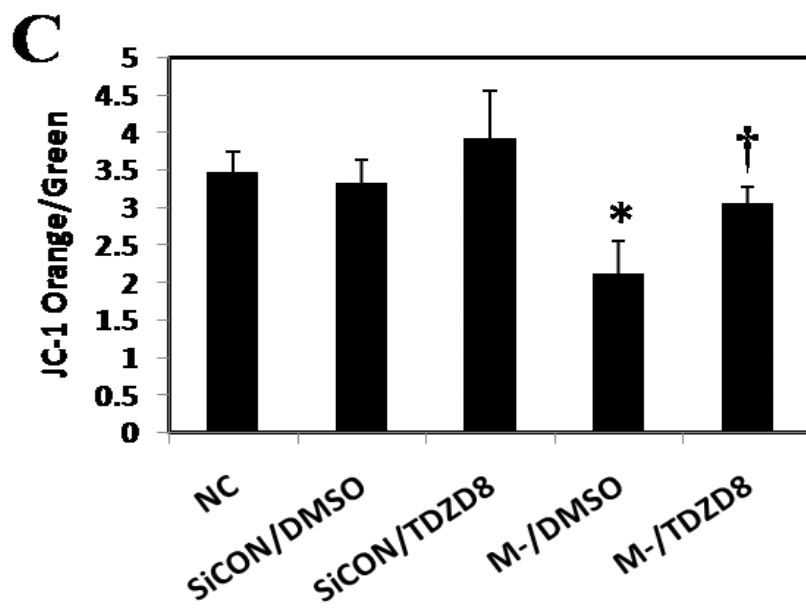


Figure 9. Effect of GSK-3 β inhibition on serum-starvation-induced apoptosis.

A-C. Each experiment was repeated three times. *, $P < 0.05$, vs. NC or SiCON.

A. C2C12 cells were co-transfected with M-cadherin-targeted siRNA and one of the plasmids (myrAkt/GSK-3 β K85R/empty vector) for 48 hours, then serum-starved for 48 hours. DNA fragmentation was measured by an ELISA. †, $P < 0.05$ vs. M-/EV.

B. C2C12 cells were transiently transfected with M-cadherin-RNAi (M-), or a non-targeted scrambled siRNA (SiCON). These were compared to normal non-transfected C2C12 cells (NC). The cells were treated with 20 μ M TDZD-8 or DMSO for the last 12 hours of siRNA transfection. Forty-eight hours after transfection, the treated and control cells were serum starved for 48 hours. The cells were harvested and DNA fragmentation was assessed by cell death ELISA. Each experiment was repeated three times. †, $P < 0.05$ vs. SiCON/TDZD8 and the M-/DMSO.

C. Mitochondria were isolated from each experimental group and stained with JC-1. The ratio of JC-1 orange/green staining was analyzed using a FACSCalibur system to measure the mitochondrial membrane potential. †, $P < 0.05$ vs. M-/DMSO.

Figure 10

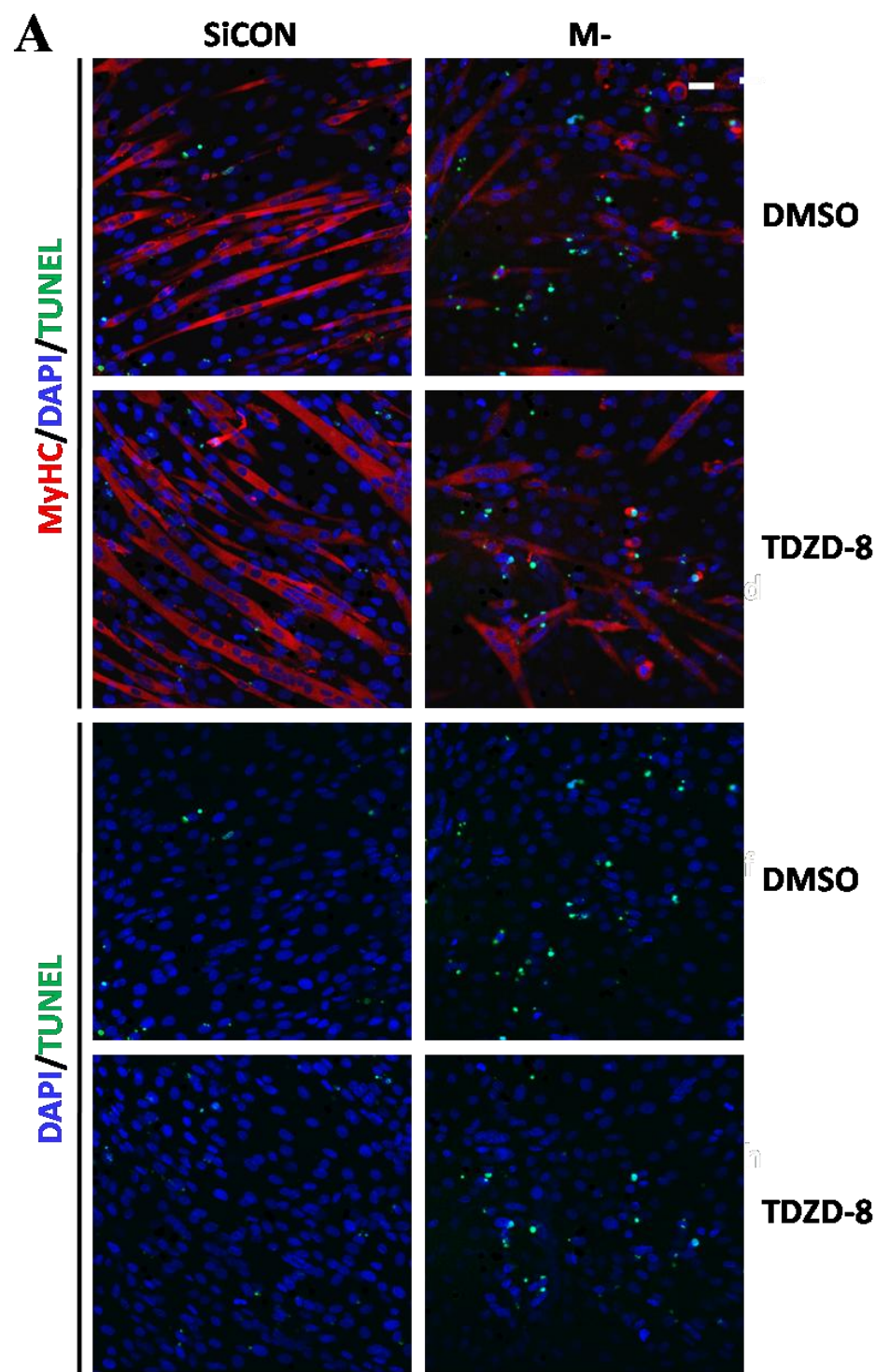


Figure 10

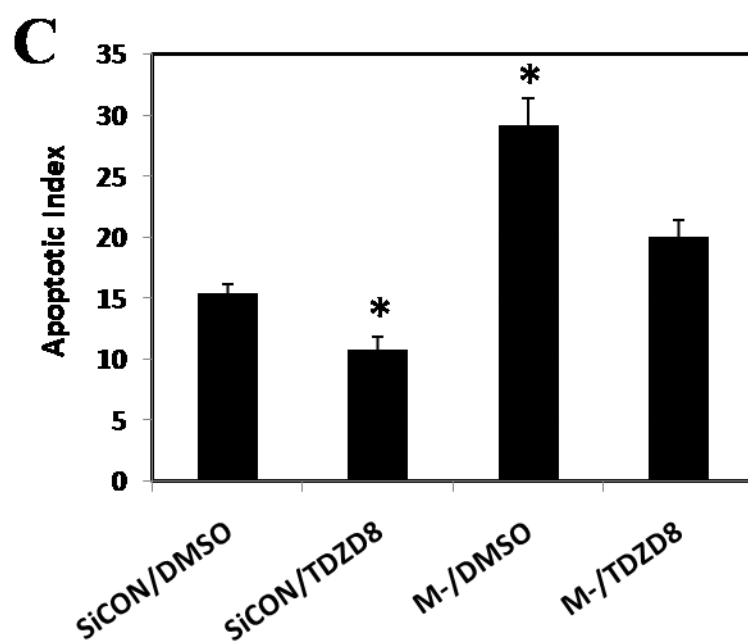
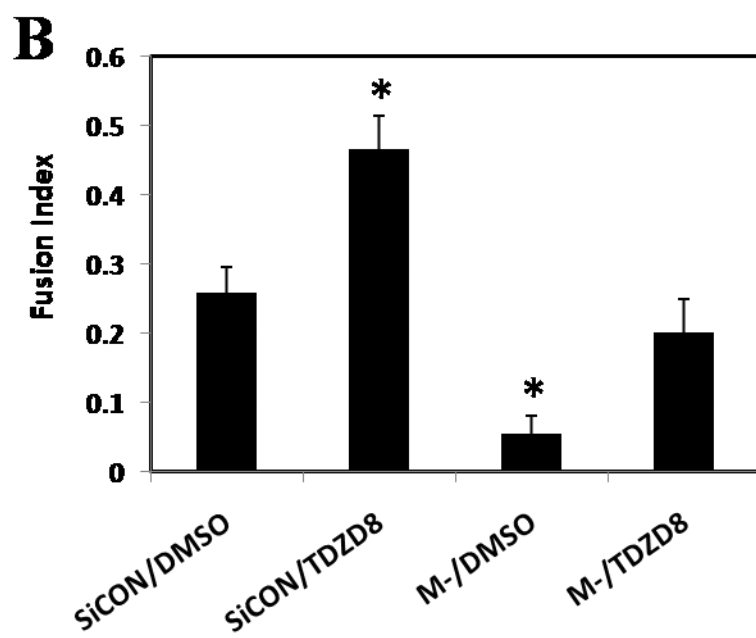


Figure 10. Effect of M-cadherin RNAi and GSK-3 β inhibition on myogenic differentiation of C2C12 myoblasts. C2C12 cells were grown on coverslips, transfected with M-cadherin-targeted (M-) or non-targeted scrambled siRNA (SiCON) for 36 hours then incubated with or without TDZD-8 for 12 hours. The cells were then cultured in differentiation medium for 48 hours.

A. Representative confocal images of C2C12 myoblasts after treatment with a combination of M-cadherin RNAi (M-) or non-targeted scrambled siRNA transfection (SiCON) and TDZD-8 or DMSO as a vehicle control. The cells were incubated in differentiation medium for 48 hours after the co-treatments. MyHC: red; DAPI: blue; TUNEL: green. Images were acquired at 20x. The scale bar = 100 μ m.

B-C. The data from three independent experiments were given as mean \pm SEM. *, $P < 0.05$ vs. SiCON/DMSO; †, $P < 0.05$ vs. M-/DMSO.

B. The myoblast fusion index was calculated for cells after each treatment described in Fig. 7A, as the ratio of the number of DAPI-positive nuclei located in the MyHC-positive myotubes (i.e., fused myoblasts) divided by the total number of nuclei in the same field.

C. The apoptotic index was calculated as the percent of total nuclei that were TUNEL positive.

Figure 11

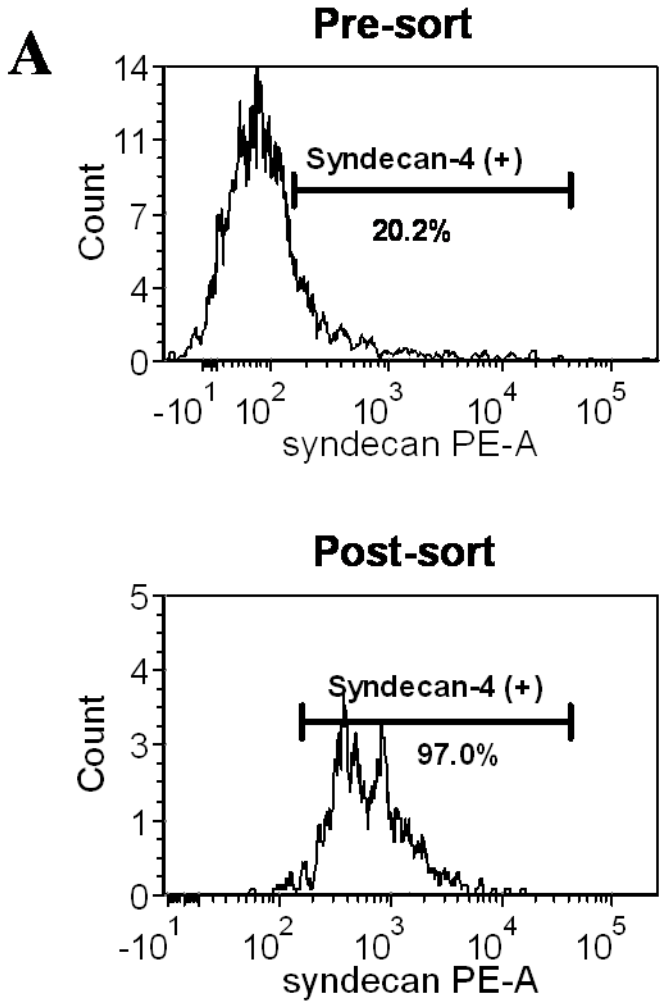


Figure 11

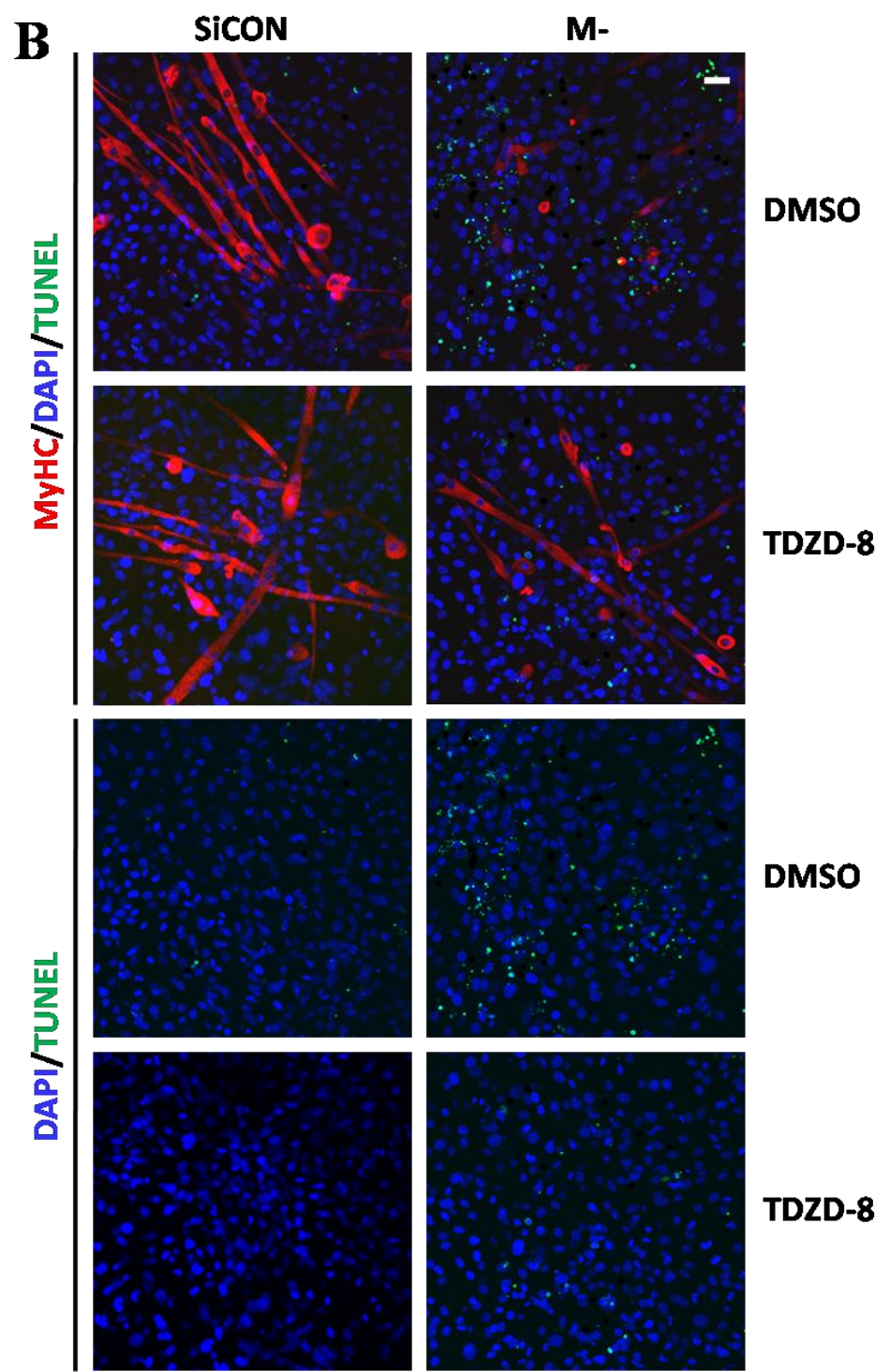


Figure 11

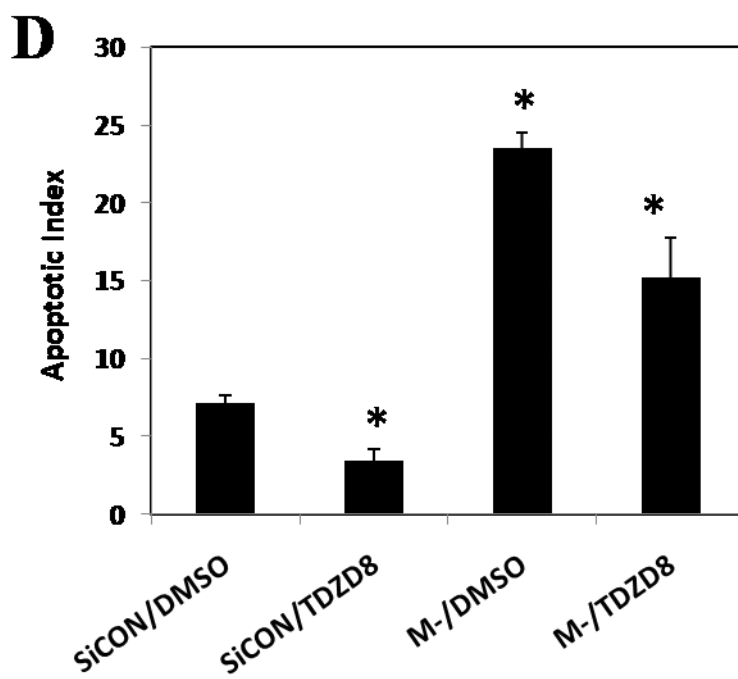
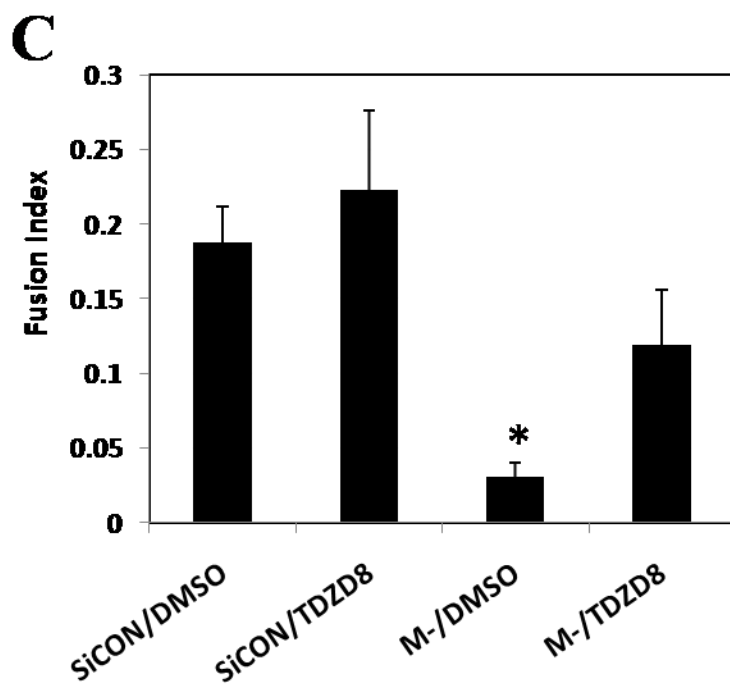


Figure 11. Effect of M-cadherin RNAi and GSK-3 β inhibition on apoptosis and myogenic differentiation of primary Syndecan-4-positive myoblasts. Primary myoblasts were isolated from hindlimb muscles of 1-week old C57BL/6 mice and Syndecan-4-positive myoblasts were purified from isolated cells by fluorescence activated cell sorting (FACS).

A. Approximately 20.2% cells of adult stem cells that were isolated from hindlimb skeletal muscles were Syndecan-4 positive (Top panel). The post-sort verification of FACS sorted cells indicated that the purity of Syndecan-4-positive cells was 97% (Bottom panel).

B. Syndecan-4-positive myoblasts were grown on coverslips and treated as described in Fig. 7. The imaging of the cells was the same as in Fig. 7A.

C-D. The data from three independent experiments were given as mean \pm SEM. *, $P < 0.05$ vs. SiCON/DMSO. †, $P < 0.05$ vs. M-/DMSO.

C. The myoblast fusion index was calculated as described in Fig. 7B.

D. The apoptotic index was calculated as described in Fig. 7C.

Figure 12

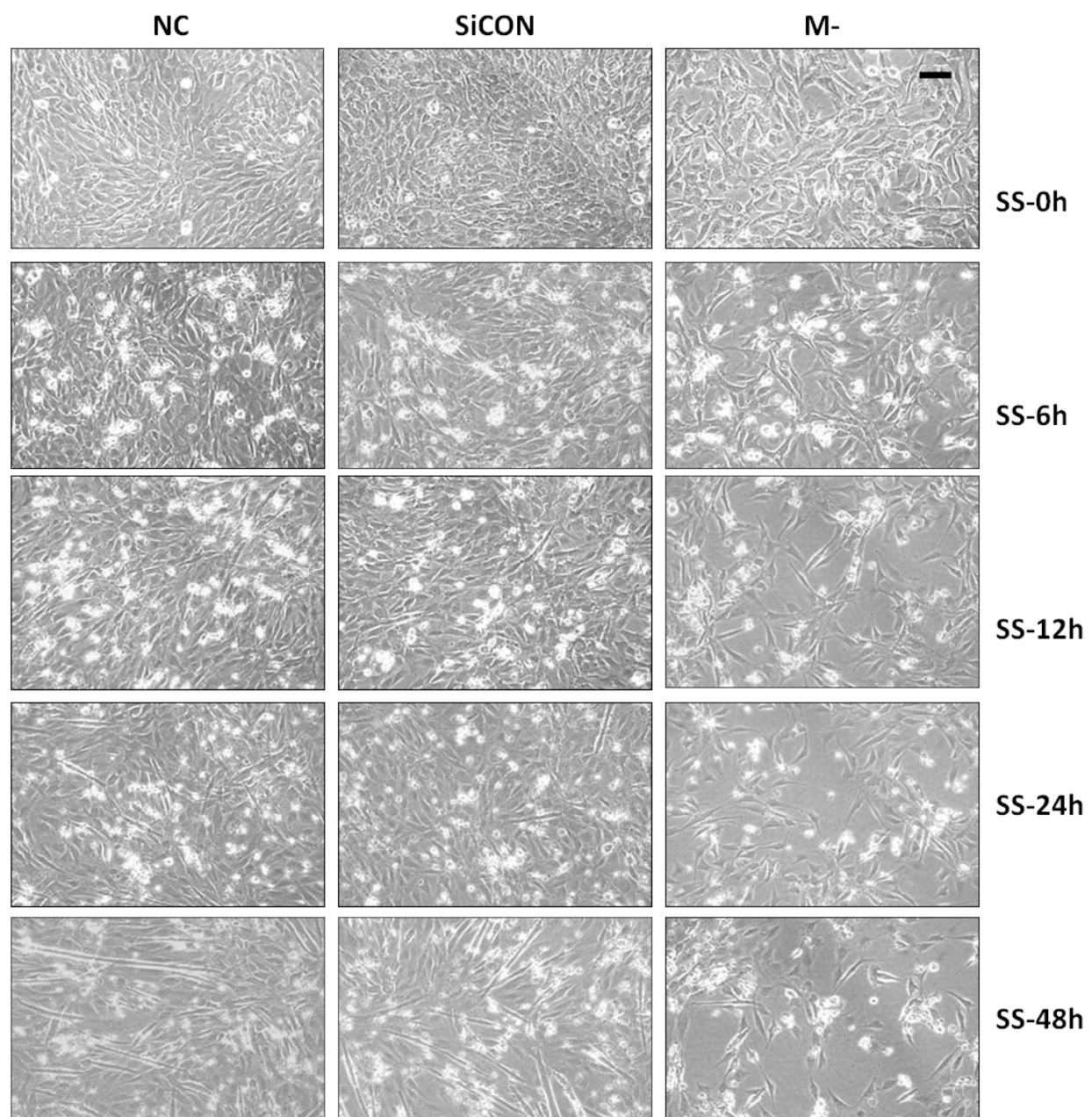


Figure 12. M-cadherin knockdown reduces cell survival. C2C12 myoblasts were plated in 6 well plates at a density of 1.7×10^5 per well. 24 hours later, the cells were transfected with M-cadherin-targeted siRNA (M-) or non-targeted scrambled siRNA (SiCON). A group of non-transfected cells with identical culture condition was used as a normal control (NC). 48 hours after transfection, the cells were treated with serum starvation for 0 (SS-0h), 6 (SS-6h), 12 (SS-12h), 24 (SS-24h), or 48 hours (SS-48h). Phase contrast images were taken at the end of each time point prior to harvesting the cells for additional analyses. The magnification of the microscope objective was 20x. The scale bar = 100 μm .

Figure 13

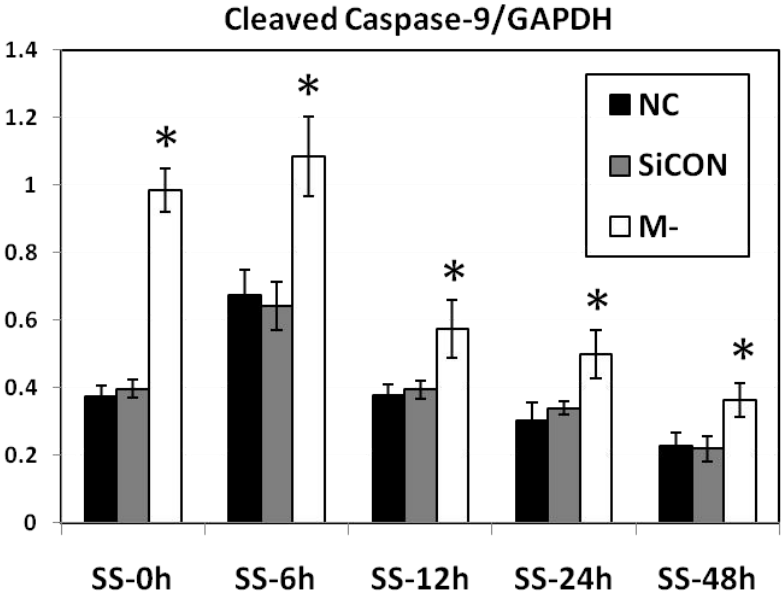
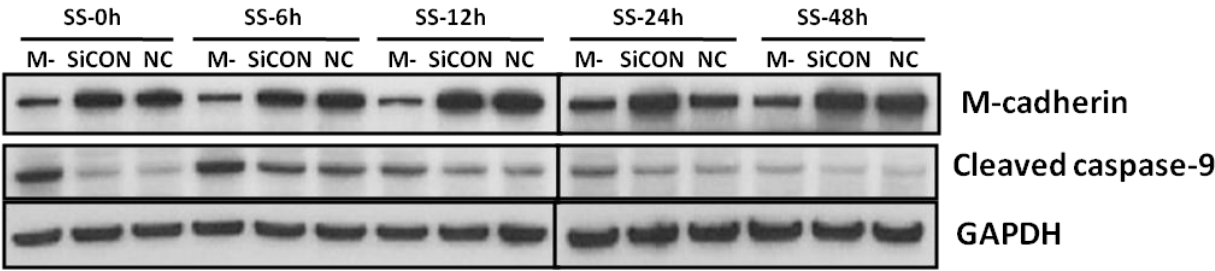


Figure 13. Immunoblotting analysis of cleaved caspases-9 in response to M-cadherin RNAi plus serum starvation. The C2C12 cells were treated as described in Figure S1. The cells were harvested in RIPA buffer at the end of each time point and processed for immunoblotting.

Top panel: Representative immunoblots are shown of cleaved caspase-9 in C2C12 myoblasts after transfections with M-cadherin-targeted siRNA (M-), non-targeted scrambled siRNA (SiCON) or in non-transfected normal control cells (NC) after 0 (SS-0h), 6 (SS-6h), 12 (SS-12h), 24 (SS-24h), or 48 hours (SS-48h) of serum starvation. GAPDH was probed as a loading control. Protein abundance of M-cadherin was detected to confirm the efficiency of M-cadherin knock-down by siRNA.

Bottom panel: Densitometric quantification of immunoblots from cleaved caspase-9 protein abundance normalized to GAPDH. Each data point (mean \pm SEM) is the mean of three independent experiments. *, a significant difference ($p < 0.05$) from the NC and SiCON control groups.

Figure 14

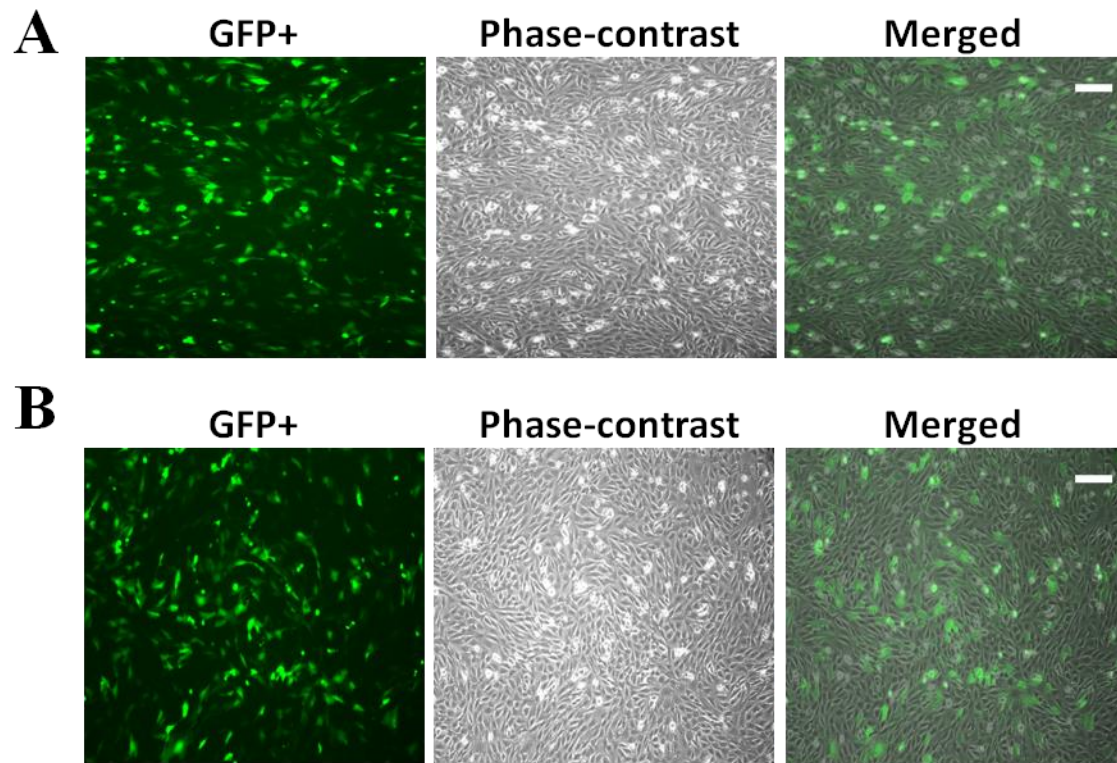


Figure 14. Determination of transient transfection efficiency in C2C12 myoblasts.

The transfection efficiency of the GSK-3 β plasmid by FuGENE 6 in was estimated to be ~20-30%. This was estimated by transfecting a pEGFP-C3 vector into C2C12 cells and counting the eGFP-positive cells in the fields 6-well plates of proliferating myoblasts with cell density ~ 70%~80% confluency were transfected with an EGFP-C3 vector (3 μ g per well) using either FuGENE 6 (Roche) (**A**) or DharmaFECT Duo (Thermo Scientific) (**B**). 48 hours after transfection, eGFP-positive cells were visualized under a Nikon Eclipse TS100 microscope connecting to an X-Cite 120 fluorescence illumination system (Lumen Dynamics). Fluorescent images as well as phase-contrast images of the same field were captured using a SPOT RT camera (Diagnostic Instruments, Inc). The magnification of objective lens was 10x. The scale bar = 200 μ m.

The transfection efficiency was estimated by counting eGFP-positive cells and total cells from ten random fields for either condition. The transfection rate was defined as number of eGFP-positive cells divided by the total number of cells within the same field. The transfection efficiency achieved by using FuGENE 6 in our experiments was 25.3% \pm 4.8%. The transfection efficiency using DharmaFECT Duo was 29.7% \pm 5.4%.

Figure 15

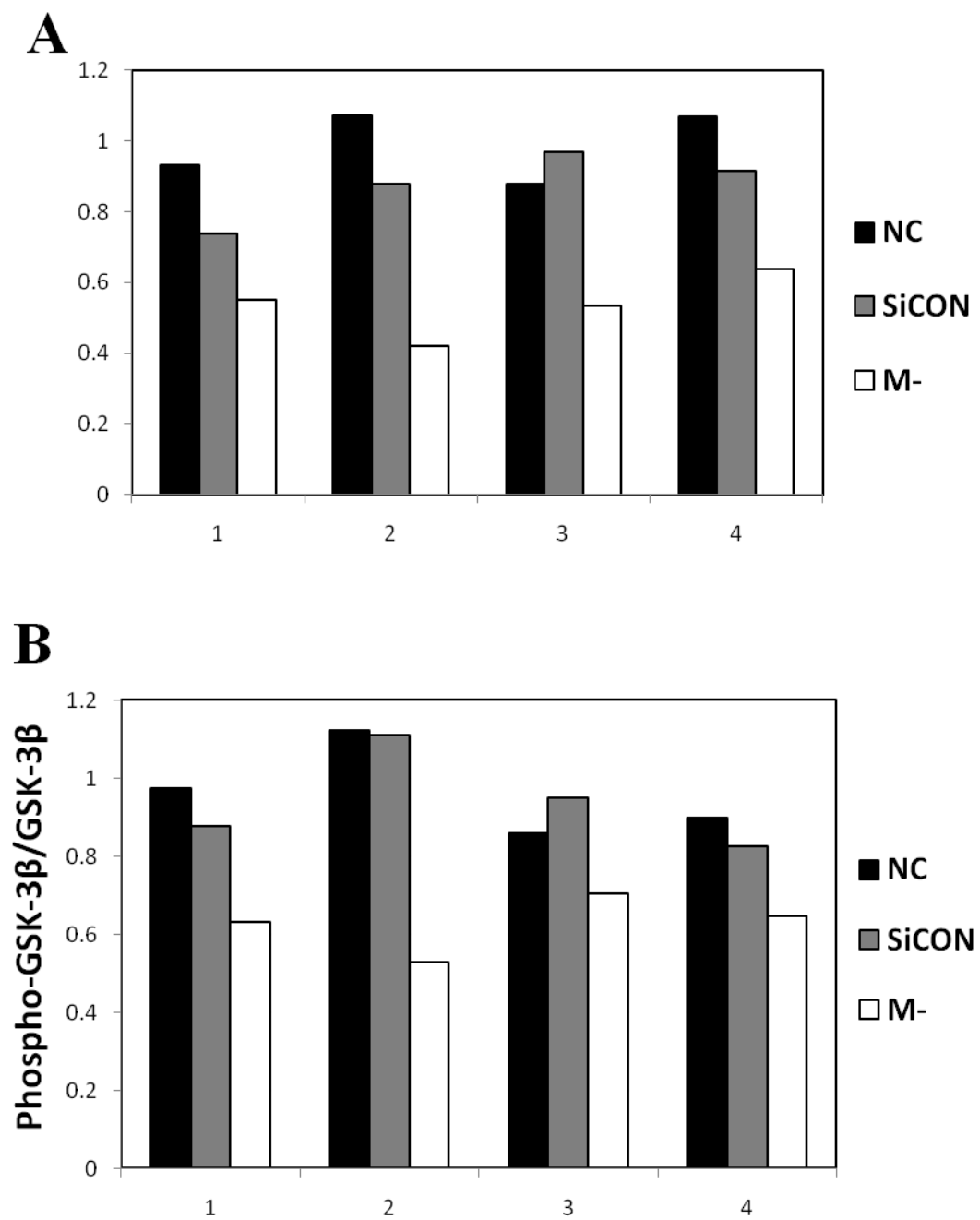
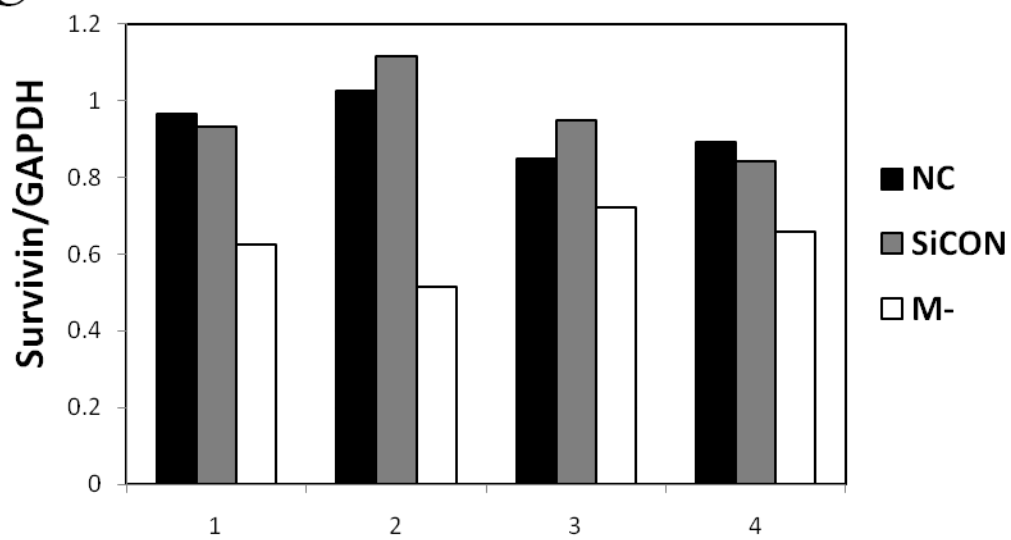


Figure 15

C



D

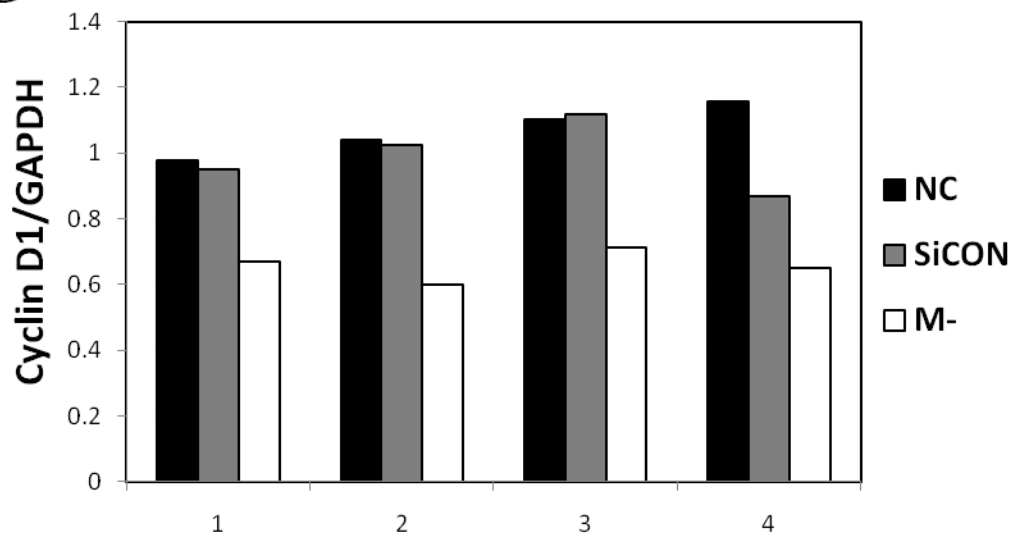


Figure 15. Densitometric analyses of immunoblot band intensities of phosphorylated and total Akt and GSK-3 β , survivin, and cyclin D1 in response to M-cadherin RNAi. C2C12 were transfected with M-cadherin-targeted siRNA (M-), non-targeted scrambled siRNA (SiCON) or in non-transfected normal control cells (NC) and harvested 48 hours after transfection. Phosphorylated protein band intensities were normalized by those of corresponding total proteins (**A**, phospho-Akt/Akt; **B**, phospho-GSK-3 β /GSK-3 β). The band intensities of survivin and cyclin D1 were normalized to GAPDH (**C**, Survivin/GAPDH; **D**, Cyclin D1/GAPDH). The data represent four separate experiments and show that the data are highly reproducible.

Figure 16

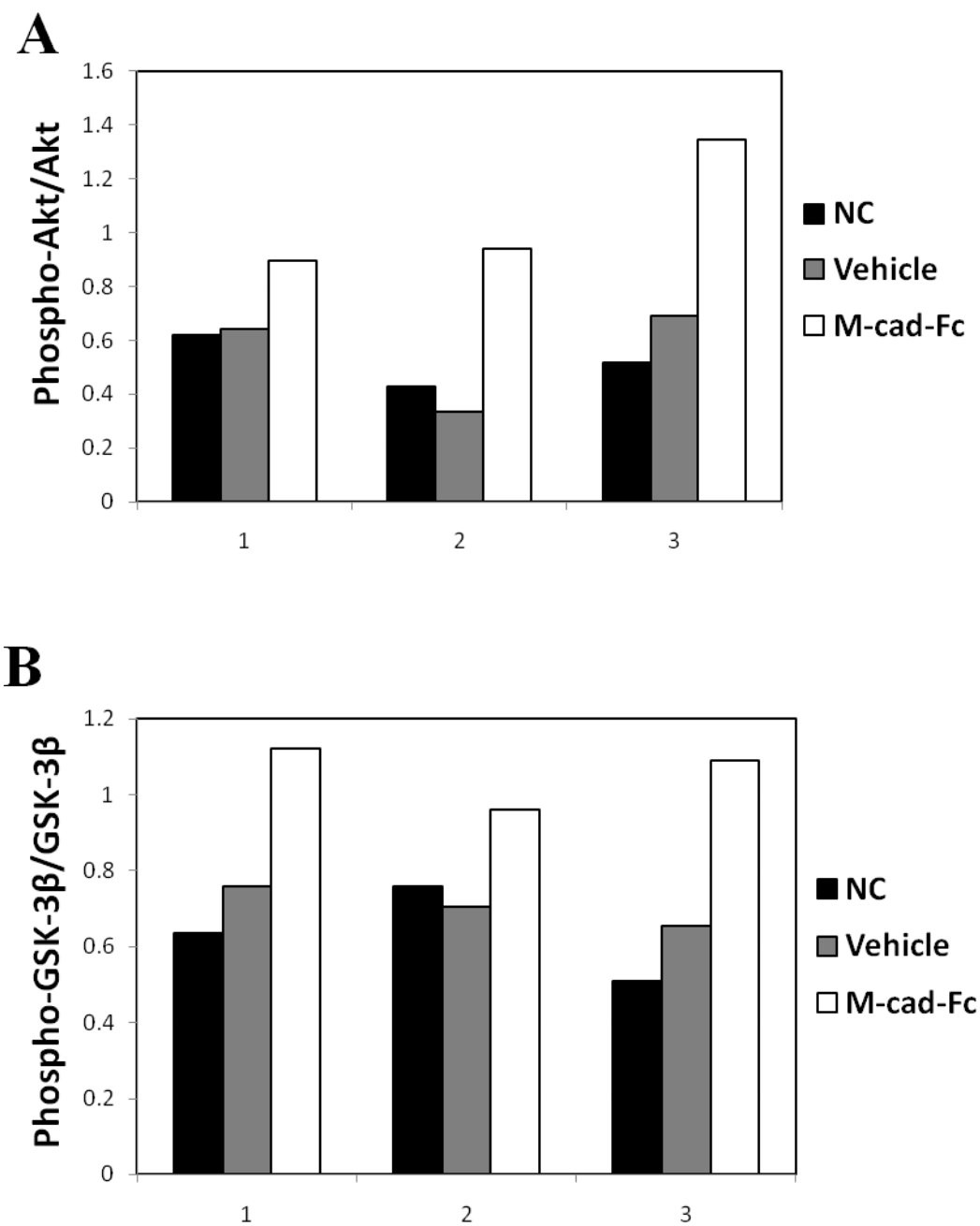


Figure 16

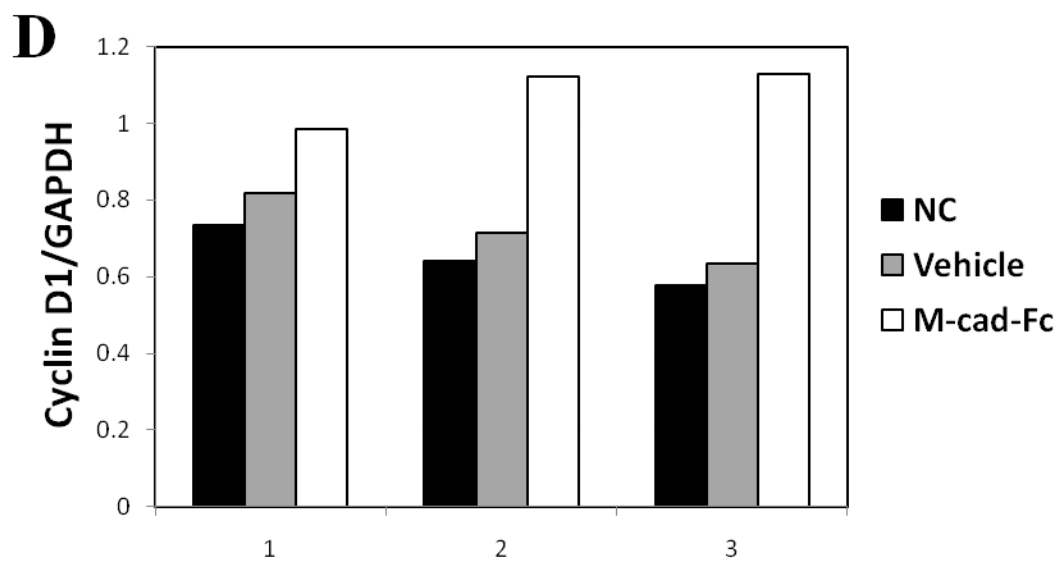
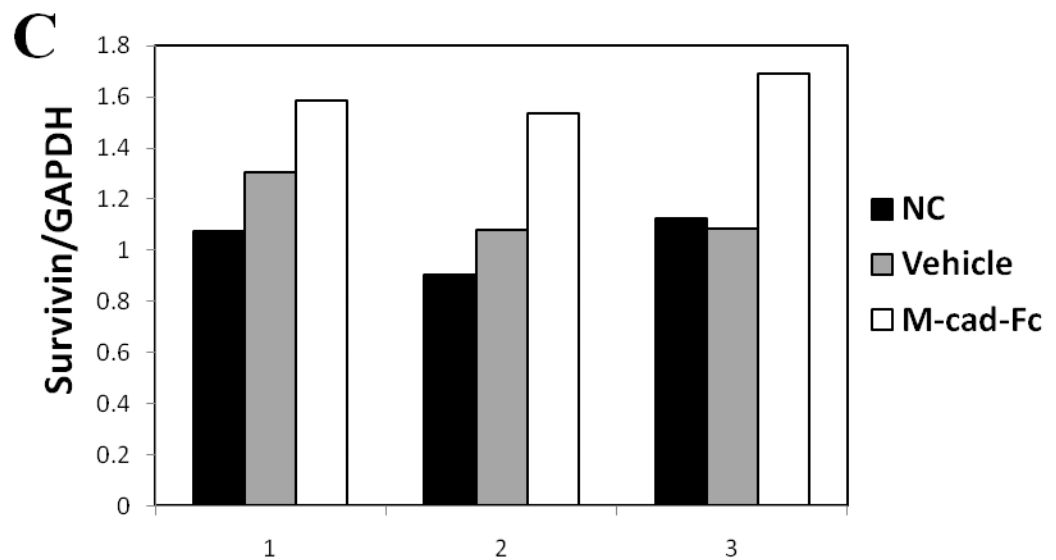


Figure 16. Densitometric analysis of immunoblot band intensities of phosphorylated and total Akt and GSK-3 β , survivin, and cyclin D1 in response to recombinant M-cadherin-Fc treatment. C2C12 myoblasts were grown in normal control (NC), vehicle control (Vehicle) or recombinant M-cadherin-Fc-coated (M-cad-Fc) dishes for 48 hours. The cells were lysed and subjected to immunoblotting analysis. Phosphorylated protein band intensities were normalized by those of corresponding total proteins (**A**, phospho-Akt/Akt; **B**, phospho-GSK-3 β /GSK-3 β). The band intensities of survivin and cyclin D1 were normalized to GAPDH. (**C**, Survivin/GAPDH; **D**, Cyclin D1/GAPDH). The data are shown from 3 separate experiments, and indicated that the results are repeatable.

Supplementary Detailed Materials and Methods

Cell Culture

C2C12 myoblasts were purchased from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco Modified Eagle's Medium (DMEM) (Invitrogen Life Technologies, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic and antimycotic mixture solution (Invitrogen), in a humidified incubator under an atmosphere of 5% CO₂ at 37°C. Serum starvation was carried out by culturing the cells in serum-free DMEM supplemented with 1% antibiotic and antimycotic mixture.

Primary myoblasts were isolated from hind limb muscles of one-week old C57BL/6 mice using a modified protocol from Dr. Margaret Goodell's lab (30). Briefly, the hindlimb muscles were minced and digested by 0.2% type II collagenase/HBSS at 37°C. The minced muscle slurry was filtered with 40 µM cell strainers and myoblasts were separated from fibroblasts and other cell components by percoll (Sigma-Aldrich Co., St Louis, MO) gradient centrifugation. The Syndecan-4-positive myoblasts were further purified from isolated cells by fluorescence activated cell sorting (FACS) using a BD FACSAria (Benton Dickson Biosciences, San Jose CA) and anti-Syndecan-4 antibody (BD) with the program of BD FACS DIVA version 6.2. The Syndecan-4-positive primary myoblasts were cultured in Ham's F10 supplemented with 20% FBS and 1% antibiotic and antimycotic mixture solution in a humidified incubator under an atmosphere of 5% CO₂ at 37°C.

Myogenic differentiation was induced by culturing the confluent C2C12 or primary myoblasts in differentiation medium composed of DMEM supplemented with 2% horse serum and 1% antibiotic and antimycotic mixture (Sigma-Aldrich).

Chemical Inhibitors

The PI3K inhibitor Wortmannin (working concentration: 200 nM) was obtained from Alexis Biochemicals (Plymouth Meeting, PA). GSK-3 inhibitor TDZD-8 (working concentration: 25 μ M) was purchased from Sigma (St. Louis, Missouri). MEK1 inhibitor PD98059 (working concentration: 50 μ M) was from Cell Signaling Technology (Danvers, MA).

Inhibition of M-cadherin expression by RNA interference

SMARTpool small interfering RNA (siRNA) targeted to M-cadherin mRNA (Life Thermo) consisted of duplex 1: sense: 5'-CGACACAGCUCUCAUCUAUUU-3', antisense: 5'-PAU AGA UGA GAG CUG UGU CGU U-3'; duplex 2: sense: 5'-UAG AUG AUA UCA ACG ACA AUU-3', antisense: 5'-PUU GUC GUU GAU AUC AUC UAU U-3'; duplex 3: sense: 5'-GAA GGA CGG CUG GUA CAG AUU-3', antisense: 5'-PUC UGU ACC AGC CGU CCU UCU U-3'; duplex 4: sense: 5'-CAG GAU GCA UAC GAC AUA AUU-3', antisense: 5'-PUU AUG UCG UAU GCA UCC UGU U-3'. The myoblasts were seeded in six-well plates 24 hours before transfection at a density of 1.7×10^5 per well. The transfection of siRNA was performed using either DharmaFECTTM-3 reagent (Life Thermo) (for C2C12 myoblasts) or Lipofectamine 2000 (Invitrogen) (for primary myoblasts) according to the manufacturer's instructions at a final siRNA duplex concentration of 100nM. The

efficacy of M-cadherin protein knockdown by RNA interference (RNAi) was confirmed by immunoblotting.

Plasmids and Transfection

The full-length mouse wild-type GSK-3 β cDNA was generated by RT-PCR. mRNA was derived from wild C2C12 myoblasts using Trizol reagent (Life Technologies, Inc.) and reverse-transcribed into cDNA using Superscript II (Life Technologies, Inc.). The forward primer was 5'-TTC CCG CGG CCG CCG CCA TGT CGG GGC GAC CGA GAA C-3', and the reverse primer was 5'-TTC CCT CTA GAT CAG GTG GAG TTG GAA GCT GA-3', with recognition sites for restriction endonucleases *NotI* and *EcoRI* included. The PCR product was cloned into the expression vector pcDNA3.1/myc-His(-) (Invitrogen). The inserted region was verified by DNA sequencing (SeqWright, Inc., Houston, TX). The constitutively active mutant of Akt (myrAkt) and the V5-tagged kinase-deficient GSK-3 β mutant (K85R) carried by vector pcDNA3 were generous gifts from Dr. Jia Luo (University of Kentucky). The wild-type GSK-3 β plasmids were transfected into the cells using FuGENE 6 reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. The co-transfection of M-cadherin siRNA with myrAkt or GSK-3 β (K85R) plasmids was carried out using DharmaFECT Duo transfection reagent (Dharmacon, Inc., Thermo-Fisher Scientific, Lafayette CO) according to the manufacturer's instructions.

Recombinant M-cadherin-Fc treatment

The recombinant M-cadherin-Fc chimera was purchased from R&D Systems. 6-well cell culture plates were pre-coated with goat anti-Fc antibody ($0.5\mu\text{g}/\text{cm}^2$ in PBS/ Ca^{2+} ; Jackson ImmunoResearch, West Grove, PA) overnight at 4°C followed by coating with recombinant M-cadherin-Fc chimera at a final concentration of $2\mu\text{g}/\text{cm}^2$ in 0.1%BSA/PBS/ Ca^{2+} for 2 hours at room temperature. The dishes were washed with PBS/ Ca^{2+} and blocked with 1% BSA/HBSS/ Ca^{2+} for 1 hour. Plates coated with anti-Fc antibody only were used as vehicle control. A number of 0.5×10^5 per well cells were plated and allowed to grow for 48 hours before being harvested for further assays.

Subcellular fractionation

The membrane fraction of C2C12 myoblasts was prepared using the Mem-PER eukaryotic membrane protein extraction reagent kit (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instruction. The nuclear protein fraction was prepared according to methods that have been previously described in our lab (31) with minor modifications. Briefly, the cells were harvested by trypsinization and incubated in lysis buffer (10mM NaCl, 1.5mM MgCl_2 , 20mM HEPES, pH 7.4, 20% glycerol, 0.1% Triton X-100, and 1mM dithiothreitol (DTT)) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, 1:100 dilution) with rotation at 4°C for 2 hours. The lysates were centrifuged at 2,500 rpm for 5 minutes at 4°C . The pellet containing nuclei portion was washed three times with lysis buffer and resuspended in nuclear lysis buffer containing 0.6M NaCl and incubated for another 2 hours at 4°C and centrifuged at $15,000 \times g$ for 15 minutes. The supernatants contain the nuclear fraction.

The mitochondrial and mitochondria-free and nuclei-free cytosolic fractions were prepared using mitochondria/cytosol reagents (BioVision, Mountain View, CA) according to the manufacturer's instruction. The concentration of the protein extracts was quantified in duplicate by Bio-Rad *Dc* Protein Assay (BioRad, Hercules, CA). The whole cell lysate was obtained by disrupting the cells with RIPA buffer (mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, 1:100 dilution) followed by centrifugation at 10,000 rpm for 5 minutes at 4°C. The supernatant was collected as the whole cell lysate.

Antibodies and immunoblotting

Antibodies specific to phosphoserine 473 Akt, total Akt, phosphoserine 9 GSK3 β , total GSK3 β , cytochrome *c*, cleaved caspase-9 and cleaved caspase-3, AIF, survivin (all the above antibodies were diluted by 1:1000) and cyclin D1 (1:2000 dilution) were purchased from Cell Signaling Technology (Danvers, MA). The anti-M-cadherin antibody (1:200 dilution) was obtained from Calbiochem (La Jolla, CA). The anti-caspase-8 antibody (1:250 dilution) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies to histone H2B (1:5000 dilution), β -tubulin (1:500 dilution) and GAPDH (1:5000 dilution) were obtained from Abcam (Cambridge, MA). The antibodies to Manganese superoxide dismutase (MnSOD) and Copper-Zinc superoxide dismutase (Cu-ZnSOD) (both diluted at 1:1000) were purchased from Millipore (Billerica, MA). The secondary antibodies for immunoblotting including goat anti-rabbit or goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) were

obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The secondary antibodies were used at dilution ranges between 1:30000 and 1:100000. Immunoblotting of the cell fractions were conducted by diluting the cell lysates in 4X NuPAGE LDS sample buffer (Invitrogen) then heating them at 70°C for 10 min. Equal amount of proteins were loaded on to a 4-12% gradient polyacrylamide gel (Invitrogen) and separated by routine get electrophoresis. The proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) and the membranes were blocked in 5% non-fat milk or 5% bovine serum albumin dissolved in Tris buffered saline with 0.05% Tween-20 (TBS-T) for 1 hour at room temperature. The membranes were probed with primary antibodies overnight at 4°C. The appropriate secondary antibody was applied to the membrane for 1 hour at room temperature. The resulting signals were developed using an enhanced chemiluminescence lighting (ECL) Western blotting detection reagent kit (GE Health Care, Piscataway, NJ). The resulting signals were visualized by exposing the membranes to X-ray films (BioMax MS-1; Eastman Kodak, Rochester, NY). Digital records were obtained from each blot and the protein bands of interest were quantified using 1-D analysis software (Eastman Kodak). The nitrocellulose membranes were stripped in a commercially available buffer (Millipore, Billerica, MA) and reprobed for β -tubulin, GAPDH, or Histone H2B as loading controls.

Immunoprecipitation

The cells were washed three times with ice-cold PBS and lysed in ice-cold buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.25% SDS, 0.1% Nonidet P-40) containing protease and phosphatase inhibitor cocktails, and centrifuged at 10,000 rpm for 5 minutes at 4°C.

The supernatants were collected and the protein concentration of each sample was determined by protein assay. 500 µg of each lysate was incubated with 10µg of mouse anti-PI3K-p85α (Abcam) or 2µg of rabbit anti-M-cadherin antibody (Santa Cruz) or mouse or rabbit IgG (Millipore) overnight at 4°C with rotation. The sample was then incubated with 20µl of Protein A/G PLUS-agarose beads (Santa Cruz) for 1 hour at room temperature with rotation. The beads were then collected by centrifugation at 3,000 rpm for 1 minute and washed three times with ice-cold lysis buffer. The bound proteins were eluted from the agarose beads in 30ul of Laemmli sample buffer (5X) at 95-100°C. The samples were centrifuged at 10,000rpm for 30 seconds and the supernatants were collected. The proteins were separated by SDS-PAGE and immunoblotted with antibodies against M-cadherin or PI3K-p85α.

Microscopy, cell morphology and the myoblast fusion index

Phase contrast microscopy. Phase contrast images of C2C12 and primary myoblast cells were captured using a Nikon Eclipse TS100 phase-contrast microscope (Nikon Instruments, Melville, NY) equipped with 10x/0.25 and 20x/0.40 phase contrast objectives and a SPOT RT camera, and analyzed with SPOT RT software (Diagnostic Instruments Inc., Sterling Heights, MI).

Immunocytochemistry and Fluorescence microscopy. Immunocytochemical assays were conducted on cells that were grown on coverslips coated with 1% gelatin. The cells were fixed with fresh 4% paraformaldehyde/PBS, permeabilized with 0.5% Triton X-100/PBS, and then blocked with 1% BSA/PBS. The coverslips were then incubated at

4°C overnight with antibodies against mouse anti-M-cadherin (1:20 dilution, Calbiochem) or mouse anti-myosin heavy chain (MyHC) (1:500 dilution, Developmental Studies Hybridoma Bank, Iowa City, IA). The primary antibodies were diluted with 1% BSA/PBS. After washing in PBS, the coverslips were incubated with the Alexa Fluor 546 goat anti-mouse IgG (H+L) (Invitrogen) at 37°C for 1 hour. The nuclei were counter-stained with 4,6-diamidino-2-phenylindole (DAPI) and the cells were mounted in VECTASHIELD mounting medium (Vector Labs) and imaged with a Zeiss LSM510 confocal on an AxioImager Z1 microscope using the Zeiss AIM software (Carl Zeiss MicroImaging, Thornwood, NY) and either a Plan-Apochromat 20x/0.8 objective or a Plan-Apochromat 63x/1.40 Oil objective, as indicated in the figure legends. The scan speed was 8 seconds and pinhole size was set at 1 Airy unit for each channel. 8-bit images (1024x1024) were sequentially acquired with equivalent settings (gain, offset, laser power, etc) for comparable intensity analysis. Tiff images (raw data) were exported and opened in Photoshop where the levels were adjusted equivalently across images.

Myoblast fusion index. The myoblast fusion index was calculated as the ratio of the number of DAPI-positive nuclei located in the MyHC-positive myotubes (i.e., fused myoblasts) divided by the total number of nuclei in the same field. This fusion index was used as a read-out of myogenic differentiation. The fusion index was obtained from 10 non-overlapping areas of each coverslip.

Apoptosis assays

DNA fragmentation. DNA fragmentation was used to assess the level of apoptosis in muscle cells using an ELISA (Roche) which assessed cytosolic nucleosomes in attached cells. Measurements for DNA fragmentation were made at an absorbance of 405nm. These methods have been described previously for our laboratory (31; 32). The data were normalized to the protein concentration of the sample.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). A TUNEL assay (In Situ Cell Death Detection Kit; Roche) was used to identify the extent of apoptotic nuclei in adherent myoblasts as reported previously (33). The nuclei of all cells were counter-stained with DAPI. The number of TUNEL- and DAPI-positive nuclei was counted in ten images that were chosen randomly from non-overlapping areas of each group. The data were presented as the apoptosis index, which was determined by dividing total number of TUNEL-positive nuclei by the total number of DAPI-positive nuclei.

As treatment with serum starvation and M-cadherin-siRNA resulted in apoptosis and cell death, some cells detached from the plates. To identify the full extent of apoptosis, and determine if M-cadherin-RNAi treatment increased the sensitivity of C2C12 myoblasts to serum starvation-induced apoptosis, we assessed apoptosis by a TUNEL assay in both adherent and floating cells in each well. The methods from Weyman's group (4) were adapted as a third approach to quantify the apoptotic cells in both attached and floating cells. Briefly, floating cells were collected by centrifugation at 500x g for 5 minutes,

washed, and fixed in 4% paraformaldehyde for 20 minutes. Thereafter, the cells were permeabilized in 0.1% Triton X-100 for 2 minutes then incubated with TUNEL reagents using the In Situ Cell Death Detection Kit (Roche). Apoptotic cells in attached cells were labeled by TUNEL staining in a similar fashion. The total percent of apoptotic cells for each well was calculated as: $(\text{the total number of TUNEL-positive attached cells} + \text{the total number of TUNEL-positive floating cells}) / (\text{total attached cells} + \text{total floating cells})$.

Cardiolipin content and mitochondrial membrane potential measurement

Nonyl acridine orange (NAO, Invitrogen) was used to determine the cardiolipin content in the inner mitochondrial membrane as an indicator of mitochondrial integrity. C2C12 myoblasts cultured on coverslips and transfected with M-cadherin-targeted or scrambled siRNA were incubated with NAO (250nM) at 37°C for 20 minutes. Fluorescence was visualized with a Nikon Eclipse E800 fluorescence microscope equipped with a Plan Fluor 100x/1.30 Oil objective and images were captured using a SPOT RT camera (Diagnostic Instruments, Inc). The exposure time was constant for all images. The fluorescence intensity was analyzed and quantified using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2011.). To measure changes in the mitochondrial membrane potential, mitochondria were isolated from M-cadherin RNAi or control cells and suspended in sucrose buffer (sucrose 220mM, mannitol 70mM, Tris HCl 10mM, EDTA 1mM, pH 7.4). The purity of mitochondria was verified by staining the mitochondria suspension with MitoTracker Deep Red 633 (Molecular Probes, Carlsbad, CA) at a final concentration of 100nM and analyzed by flow cytometry. This fluorescent

dye diffuses passively into intact and respiring mitochondria. Debris was excluded by appropriate gating strategies during flow cytometry analysis. The mitochondrial membrane potential ($\Delta\psi_{mt}$) was measured by staining the mitochondria with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Molecular Probes) at a final concentration of 10ug/ml. The staining results of JC-1 orange/green were analyzed using a FACSCalibur system (BD Bioscience) using Cell Quest Pro. 4.0 Software. 100,000 gated events were collected for each sample.

CHAPTER 3:

M-cadherin-Mediated Signaling Modulates Phosphorylation of β -catenin N-terminus and Promotes Myogenic Differentiation in a TCF/LEF-Independent Manner

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ABSTRACT

β -catenin is an essential molecule in both cadherin-mediated cell-cell adhesion and canonical Wnt signaling. The phosphorylation of β -catenin by GSK-3 β at residues serine31/37/threonine41 of N-terminus regulates its stability and its role in canonical Wnt signaling. Recent work from our lab found that M-cadherin-mediated signaling protects myoblasts against apoptosis and promotes myogenic differentiation by suppressing GSK-3 β activation. In this study, we further investigated the possibility that the N-terminal phosphorylation of β -catenin was regulated by M-cadherin and that this regulation mediates M-cadherin's role in promoting myogenic differentiation. We demonstrated that knockdown of M-cadherin expression by RNA interference (RNAi) in C2C12 myoblasts significantly enhances the phosphorylation of β -catenin at ser33/37/thr41 and decreased the protein abundance of ser37/thr41-unphosphorylated active β -catenin. This effect can be relieved by LiCl treatment. Furthermore, M-cadherin RNAi promotes LiCl or Wnt-3a-induced TCF/LEF transcription activity but significantly blunts the myogenic induction by LiCl or Wnt-3a treatment. Knockdown of β -catenin expression by RNAi in C2C12 myoblasts also significantly decreased myogenic induction by LiCl treatment. Forced expression of phosphorylation-resistant mutated β -catenin (S33Y- β -catenin) failed to enhance myogenic differentiation but partially rescued the myogenic and apoptotic phenotype caused by M-cadherin RNAi. These data show that M-cadherin-mediated signaling attenuates β -catenin phosphorylation at Ser31/37/Thr41 by GSK-3 β and this regulation has a positive effect on myogenic differentiation induced by canonical Wnt signaling independent of TCF/LEF transcription

activity. Our findings provide a novel mechanism by which M-cadherin mediates myogenic differentiation.

INTRODUCTION

β -catenin is a key transcription cofactor within the T cell factor/lymphoid enhancer factor (TCF/LEF) family of DNA binding proteins in the Wnt signaling pathway. It is also a central structural adaptor protein linking cadherins to the actin cytoskeleton in intercellular adherens junctions (1; 2). Without Wnt signaling, a sequential phosphorylation of β -catenin by serine/threonine kinases casein kinase I α (CKI α) at serine45 and glycogen synthase kinase-3 β (GSK-3 β) at serine 33/37/threonine 41 results in ubiquitination and degradation of cytoplasmic β -catenin. Activation of Wnt signaling leads to inactivation of GSK-3 β , resulting in accumulation of cytoplasmic β -catenin, which translocates to nucleus and binds to the TCF/LEF transcription factors to induce target gene expression. As a result, the key factors in β -catenin signaling include its stabilization and accumulation in the cytoplasm (3-5). β -catenin-mediated canonical Wnt signaling is crucial to muscle development (6; 7). One of the key mechanisms by which the β -catenin/Wnt pathway regulates myogenesis, is to induce the expression of basic helix-loop-helix (bHLH) myogenic regulatory factors (MRFs) such as Myf5 and MyoD (8; 9). The β -catenin/Wnt pathway is also essential for the myogenic specification of muscle-derived CD45-positive stem cells after injury (10) and differentiation of multipotent cells into myogenic cells (11; 12).

It is well established that a key role of the cadherins is to inhibit β -catenin/canonical Wnt signaling (13-17). In addition to its direct binding and mechanical sequestering effect (18; 19), reports from several labs show that cadherins may also modulate β -catenin signaling via an adhesion-independent mechanism (13; 16; 17). Recent data from Gorttadi and colleagues suggest that E-cadherin directly promotes β -catenin phosphorylation at N-terminus by GSK-3 β in S2480 colon carcinoma cell lines (20), which in turn has an inhibitory effect on Wnt signaling. This provides a potential mechanism to explain how cadherins regulate β -catenin signaling function at least in cancer cells, although it is not known if this signaling would be similar in muscle cell lines.

As a member of the classical cadherin family of transmembrane glycoproteins that mediates calcium-dependent homophilic cell-cell adhesion, M-cadherin has been shown to be crucial in mediating myoblast alignment and fusion (21; 22), and it is required for myogenic differentiation (23). We have recently reported that M-cadherin-mediated signaling suppresses GSK-3 β activation and protects myoblasts against intrinsic mitochondrial-dysfunction associated apoptosis during myogenic differentiation (24). However, the role for M-cadherin to regulate β -catenin phosphorylation at N-terminus by GSK-3 β in myoblasts and the potential impact that this regulation might confer to myogenic differentiation is unknown.

In the present study, we investigated the regulatory effect of M-cadherin signaling on β -catenin N-terminal phosphorylation by GSK-3 β and the impact of this regulation on myogenic differentiation. We show that knockdown of M-cadherin expression promotes TCF/LEF transcription activity but decreases the availability of N-terminal unphosphorylated signaling-active β -catenin in confluent C2C12 myoblasts. This effect

can be reversed by LiCl treatment. Furthermore, both M-cadherin and β -catenin RNAi abrogated the myogenic and fibrotic induction by LiCl treatment. Forced expression of a phosphorylation-resistant mutated β -catenin induces a dramatic increase in TCF/LEF transcription activity but failed to induce myogenic differentiation by itself. However, it partially rescued the myogenic and apoptotic phenotype caused by M-cadherin RNAi. Together, these results suggest that M-cadherin signaling suppresses GSK-3 β -dependent β -catenin N-terminal phosphorylation and promotes myogenic differentiation in a TCF/LEF-independent manner.

MATERIALS AND METHODS

Cell Culture

C2C12 myoblasts were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic and antimycotic mixture solution (Invitrogen), in a humidified incubator under an atmosphere of 5% CO₂ at 37°C. Myogenic differentiation was induced by culturing the myoblasts in differentiation medium composed of DMEM, 2% horse serum and 1% antibiotic and antimycotic mixture. Recombinant Wnt-3a (working concentration: 50ng/ml) and recombinant Dickkopf-related protein (DKK-1, working concentration: 200ng/ml) were purchased from R&D Systems (Minneapolis, MN) for cell treatment. Lithium chloride (LiCl; working concentration: 20mM) was purchased from Sigma-Aldrich (St. Louis, MO).

Inhibition of M-cadherin and β -catenin expression by RNA interference

*SMART*pool small interfering RNA (siRNA) targeted to M-cadherin and β -catenin mRNAs were purchased from Dharmacon/Thermo Scientific (Lafayette, CO). The M-cadherin-targeted siRNA consisted of duplexes targeting the following sequences: 5'-CGACACAGCUCUCAUCUAU-3'; 5'-GAAGGACGGCUGGUACAGA-3'; 5'-GAGCAAACGCUGAACGUCA-3'; 5'-UCGACGAGCACACGGGAGA-3'. The β -catenin-targeted siRNA consisted of duplexes targeting the following sequences: 5'-GAACGCAGCAGCAGUUUGU-3'; 5'-CAGCUGGCCUGGUUUGAUA-3'; 5'-GCAAGUAGCUGAUUAUUGAC-3'; 5'-GAUCUUAGCUUAUGGCAAU-3'. The myoblasts were seeded in six-well plates 24 hours before transfection at a density of 1.7×10^5 per well. The transfection of siRNA was performed using either DharmaFECTTM-3 reagent (when transfected alone) or DharmaFECTTM-Duo reagent (when co-transfected with plasmids) (Thermo Scientific) according to the manufacturer's instructions at a final siRNA duplex concentration of 100nM.

Plasmids and Transfection

The phosphorylation-resistant mutated β -catenin plasmid (pcDNA3-S33Y- β -catenin), β -catenin/Wnt reporter plasmid super 8x TOPFlash, and its mutated control plasmid, super 8x FOPFlash, were purchased from Addgene (Cambridge, MA). The plasmids were extracted from bacteria and purified using EndoFree plasmid Maxi Kit (Qiagen). The plasmids were transfected into the cells using either FuGENE 6 reagent (Roche) (when transfected alone) or DharmaFECTTM-Duo (Thermo Scientific) (when co-transfected with other plasmids or with siRNA) according to the manufacturer's

instructions.

Luciferase reporter assay

Cells were transfected with 2µg of super 8xTOPFlash or FOPFlash reporter plasmid and allowed to grow for 24-36 hours before further treatment. And at the end of treatment, the cells were harvested in lysis buffer and 20 µl of each lysates was monitored for luciferase activity using a Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Light units were measured using Berthold Autolumat Plus tube luminometer (Berthold Technology, Germany). The TCF/LEF transcription activity was determined as ratios of TOPFlash to FOPFlash luciferase activity from triplicate experiment.

Cell surface biotinylation assay

A cell surface biotinylation assay was used to quantify the subcellular (membrane-bound vs. membrane-free) protein abundance of β -catenin at different cell densities according to published protocols (25) with minor modifications. Briefly, the cells were washed three times with ice-cold PBS/Ca-Mg (138mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 9.6 mM Na₂HPO₄, 1mM MgCl₂, 0.1 mM CaCl₂) and incubated with EZ link Sulfo-NHS-SS-biotin (1mg/ml, Pierce) in PBS/Ca-Mg for 30 min at 4°C. The reaction was quenched by two washes with ice-cold 100 mM glycine in PBS/Ca-Mg for 10 min each and three times washes with ice-cold PBS/Ca-Mg. The cells was scraped from the dishes and lysed in ice-cold RIPA buffer containing protease inhibitor cocktail (Sigma, 1:100 dilution). The cell lysates were centrifuged at 15,000rpm for 30 min at 4 °C, and aliquots of supernatants were incubated with streptavidin beads (Sigma, 25 µl

of beads for 500ug/500µl of aliquot) for 1 hour at 4°C with rotation. The beads were washed three times with ice-cold RIPA buffer. The bound proteins were eluted with 30ul of Laemmli sample buffer (5X) for 5 min at 95-100°C. The whole cell lysates and the unbound fractions were processed through the routine sample preparation procedure for immunoblotting.

Antibodies and immunoblotting

Antibodies that were specific to phosphorylated β -catenin (serine31/37/threonine41) and cyclin D1 were purchased from Cell Signaling Technology (Danvers, MA). An antibody to M-cadherin was from Calbiochem (La Jolla, CA). The antibody to the serine37/threonine41-unphosphorylated active β -catenin was purchased from Millipore (Billerica, MA). The antibody to total β -catenin was purchased from Sigma (St. Louis, MO). Antibodies to Axin2, Troponin T, and GAPDH were obtained from Abcam (Cambridge, MA). All the secondary antibodies for immunoblotting including goat anti-rabbit or goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Immunoblotting of cell lysates were conducted according to established protocols in our lab (24). Briefly, the cell lysates were diluted in 4X NuPAGE LDS sample buffer (Invitrogen) and boiled at 70°C for 10 min. Equal amount of proteins were loaded in a 4-12% gradient polyacrylamide gel (Invitrogen) and separated by electrophoresis. The proteins were transferred to a nitrocellulose membrane (Bio-Rad) and the membranes were blocked in 5% non-fat milk dissolved in Tris buffered saline with 0.05% Tween-20 (TBS-T) for 1 hour at room temperature and then probed with primary antibodies overnight at 4°C. This was followed by incubation with secondary

antibodies for 1 hour at room temperature. The resulting signals were developed using Amersham enhanced chemiluminescence lighting (ECL) western blotting detection reagent kit (GE Health Care) and exposed to films. The protein signals were captured with a digital camera (Kodak 290) with the KODAK 1D molecular imaging software. Densitometric analysis of immunoblot intensities was done using KODAK 1D molecular imaging software.

Cell Imaging

For immunocytochemistry, cells were grown on coverslips coated with 1% gelatin. Cells ready for immunocytochemical staining were fixed with fresh 4% paraformaldehyde/PBS, permeabilized with 0.5% Triton X-100/PBS, blocked with 1% BSA/PBS. Primary antibodies were diluted with 1% BSA/PBS. The antibodies that were used for cell imaging were: mouse anti- β -catenin antibody (Sigma-Aldrich), rabbit anti- β -catenin antibody (Cell Signaling), mouse anti-serine37/threonine41-unphosphorylated active β -catenin antibody (Millipore), mouse anti-myosin heavy chain (MyHC) antibody (Developmental Studies Hybridoma Bank), and rat anti-ER-TR7 antibody (Santa Cruz Biotechnology). The coverslips were incubated with the appropriate primary antibody at 4°C overnight followed by incubation with the corresponding Alexa Fluor 546 or 488 goat anti-mouse, anti-rabbit, or anti-rat IgG (H+L) (Molecular Probes) secondary antibody at 37°C for 1 hour. The nuclei were counter-stained with 4,6-diamidino-2-phenylindole (DAPI) and the cells were mounted in VECTASHIELD mounting medium (Vector) and imaged with a Zeiss LSM510 confocal laser scanning microscope using AIM software (Carl Zeiss MicroImaging). The exposure time was 8 seconds and the hole size was set as 1.

Differentiation Index and Fusion Index

After 48-hours in differentiation the cells underwent the immunofluorescent staining of MyHC and nuclei as described above. The numbers of DAPI-positive nuclei were counted from 10 non-overlapping areas of each coverslip. The differentiation index was determined by calculating the ratio of the number of nuclei in MyHC-positive cells divided by the total number of nuclei in the same field. The fusion index was calculated as the ratio of the number of nuclei located in myotubes divided by the total number of nuclei in the same field (24; 26).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

A TUNEL assay (In Situ Cell Death Detection Kit; Roche) was used to identify the extent of apoptotic nuclei in adherent myoblasts as reported previously (27; 28). The nuclei of all cells were counter-stained with DAPI. The number of TUNEL- and DAPI-positive nuclei was counted in ten images that were chosen randomly from non-overlapping areas of each group. The data were presented as the apoptotic index, which was determined by dividing total number of TUNEL-positive nuclei by the total number of DAPI-positive nuclei.

Statistical analyses

The results are given as means \pm standard error of mean (SE). Statistical analyses were performed using the SPSS 13.0 software package. A one-way analysis of variance (ANOVA) was used to compare differences in all measured variables. $P < 0.05$ was considered statistical significant.

RESULTS

GSK-3 β -dependent phosphorylation of β -catenin N-terminus is reduced but TCF/LEF transcription activity is also down-regulated in C2C12 myoblasts at high cell density. β -catenin signaling and Wnt activity are regulated by cell confluence in various types of cells (29; 30). The expression level and pattern of cadherin proteins vary as the cell density changes. Cadherin is central partner that binds to β -catenin and in turn, regulates both the subcellular distribution and transcription activity of β -catenin. Thus to examine the phosphorylation status of β -catenin N-terminus by GSK-3 β , mouse C2C12 mouse myoblasts were seeded at either $2.0 \times 10^3/\text{cm}^2$ to obtain a low density (~20-30% confluent) or $2.1 \times 10^4/\text{cm}^2$ to reach a high cell density (~100% confluent) within 48 hours. Immunoblot analyses showed that the protein abundance of phosphorylated β -catenin at residues serine33/37/threonine41 of N-terminus decreased, and those of “active” serine37/threonine41-unphosphorylated β -catenin and total β -catenin increased when cells were confluent (Figure 17A). In addition, the protein abundance of two target proteins for canonical Wnt signaling, Axin2 and cyclin D1, were decreased. We next examined the subcellular expression pattern of β -catenin at different cell densities. As shown in left panel of Figure 17B, β -catenin was located in both the nuclei and the cell cytoplasm, especially in perinuclear regions in cells at a low density. In contrast, β -catenin was detected in the cytoplasm and also more prominently in adjacent cell-cell contacting regions when the cells were confluent (Figure 17B, right panel). The protein abundance of membrane-bound versus membrane-free β -catenin was then evaluated via a cell surface biotinylation assay. As shown in Figure 17C, the ratio of membrane-bound/membrane-free β -catenin was significantly higher in cells that were grown at a

high cell density as compared with cells grown at a low density. This finding is consistent with the data showing that a large portion of β -catenin translocates to cell-cell adhesive membrane regions in confluent cells (Figure 17B). To examine the difference in canonical Wnt signal activity in cells at different densities, we plated and grew cells transfected with either the TOPFlash reporter vector or its mutant control FOPFlash vector, in either low or high cell densities as described in Materials and Methods. As shown in Figure 17D, TCF/LEF transcription activity, as determined from the ratio of luciferase activity in TOPFlash vector-transfected cells normalized by that of FOPFlash vector-transfected cells, was significantly less in cells that were grown at high density, as compared with cells that were grown at a low density. However, in spite of the decreased TCF/LEF transcription activity, myogenic differentiation, as shown by immunofluorescent staining of myosin heavy chain (MyHC)-positive myotubes, was clearly enhanced in confluent C2C12 myoblasts as compared with cells that were grown at a low cell density in differentiation medium for 48 hours (Figure 17E). These data suggest that phosphorylation of β -catenin N-terminus by GSK-3 β is suppressed, and TCF/LEF transcription is also down-regulated when C2C12 myoblasts become confluent. The high myogenic outcome of confluent myoblasts is independent of TCF/LEF transcription activity.

M-cadherin RNAi increases phosphorylation of β -catenin N-terminus but has positive effect on TCF/LEF transcription activity. We have reported previously that M-cadherin-mediated signaling suppresses GSK- β activation in confluent myoblasts (24). To further investigate if M-cadherin-mediated signaling modulates GSK-3 β -dependent N-terminal phosphorylation of β -catenin, and the impact of this potential

modulation on TCF/LEF transcription activity in confluent C2C12 myoblasts, we inhibited M-cadherin expression in confluent C2C12 myoblasts via M-cadherin RNA interference (RNAi) and subsequently treated the cells with lithium chloride (LiCl), an established GSK-3 β inhibitor and Wnt activator. LiCl inhibits GSK-3 β activity by competing with ATP in the ATP-binding site of the kinase via directly competing with magnesium and by increasing the inhibitory phosphorylation of Serine 9 residue of GSK-3 β (31). The protein abundance of phospho- and total β -catenin as well as the unphosphorylated active β -catenin (ABC) were analyzed by immunoblotting. As shown in Figures 18A and 18B, as the protein abundances of both total β -catenin and unphosphorylated active β -catenin were significantly increased in LiCl-treated cells, M-cadherin RNAi aborted this effect (Figure 18B, middle and bottom panels). In addition, M-cadherin RNAi alone significantly increased the phosphorylation of β -catenin N-terminus and LiCl treatment effectively reversed this induction (Figure 18B, top panel). Furthermore, compared with the scrambled siRNA-transfected group, M-cadherin RNAi alone did not alter the protein abundance of total β -catenin (Figure 18B, bottom panel) but decreased the abundance of unphosphorylated active β -catenin. This effect could be rescued by LiCl treatment (Figure 18B, middle panel). These results suggest that M-cadherin-mediated signaling attenuates phosphorylation of β -catenin at N-terminus and maintains unphosphorylated active β -catenin in a GSK-3 β -dependent manner. We further investigated the subcellular expression pattern of the unphosphorylated active β -catenin in confluent myoblasts and the effect of M-cadherin RNAi and/or LiCl treatment on β -catenin via an immunofluorescence assay. Figure 18C shows that without LiCl treatment, β -catenin was localized primarily to cell-cell contact regions and in the cell's

cytoplasm. Staining patterns with the anti-ABC antibody demonstrated that unphosphorylated active β -catenin mainly resides in the cytoplasmic space, but not at cell-cell contacting membrane regions. In addition, upon LiCl treatment, unphosphorylated active β -catenin but not total β -catenin translocates into nuclei (Figure 18C, (i) and (ii)). These results suggest that the unphosphorylated active form of β -catenin is most responsive to LiCl-induced Wnt activation. However, M-cadherin RNAi did not alter the expression pattern of ABC and total β -catenin with or without LiCl treatment (Figure 18C, (iii) and (iv)). We further examined the change in TCF/LEF transcription activity in response to M-cadherin RNAi. As shown in Figure 18D, in spite of the decreased protein abundance of ABC, luciferase activity increased indicating that TCF/LEF transcription activity was increased in cells transfected with M-cadherin-targeted siRNA compared with that in control groups. To further verify M-cadherin's role in regulating TCF/LEF transcription activity, we co-transfected the cells with M-cadherin-targeted siRNA and TOPFlash/FOPFlash-reporter plasmids and then treated the cells with either LiCl or recombinant Wnt-3a for 12 hours. The luciferase activity was measured in cells at the end of treatments. As shown in Figure 18E, both LiCl and Wnt-3a treatment induced a significant increase in TCF/LEF transcription activity and both inductions were significantly enhanced in cells transfected with M-cadherin-targeted siRNA.

M-cadherin RNAi blunts myogenic and fibrotic induction by LiCl treatment. To further examine the effect of M-cadherin RNAi on the phenotype of myoblasts induced by LiCl treatment, C2C12 myoblasts transfected with either an M-cadherin-targeted or a scrambled non-targeted siRNA then treated with LiCl or recombinant Wnt-3a for 12

hours. This was followed by culture in differentiation medium for 48 hours to induce myogenic differentiation. The cells were then harvested for immunoblotting analysis. As shown in Figure 19A, the protein abundance of Troponin T, an established marker for terminal myogenic differentiation (32; 33), was significantly increased in LiCl or Wnt-3a-treated cells compared with vehicle control cells. However, M-cadherin RNAi significantly attenuated the induction of Troponin T expression in both vehicle and LiCl/Wnt-3a-treated cells. Further morphological examination via immunofluorescent staining of MyHC-positive myotubes and nuclei (Figure 19B, top panel) showed that LiCl treatment induced a significant increase in both myogenic differentiation and fusion as determined by calculating the differentiation and fusion indexes. The myogenic differentiation and fusion inductions were both significantly blunted by M-cadherin RNAi (Figure 19B, middle and bottom panels). Since dysregulated increases in Wnt signal could potentially skew the myogenic progenitor cells into fibrotic lineages (34; 35), we further examined the expression of a fibroblast marker, ER-TR7 (34; 36) in cells after the various treatments. As shown in Figure 19C, LiCl treatment induced a substantial increase in the expression of ER-TR7 in C2C12 myoblasts while M-cadherin RNAi effectively abrogated this induction. These results suggest that M-cadherin plays a positive role in mediating myogenic and fibrotic induction by Wnt signal activation caused by LiCl treatment.

β -catenin RNAi and DKK-1 treatment reverses myogenic and fibrotic induction by LiCl treatment. To further verify the role of β -catenin/Wnt signaling in mediating myoblast phenotype induced by LiCl treatment, we treated the cells either with β -catenin RNAi to inhibit β -catenin expression, or with DKK-1, an established antagonist of

canonical Wnt signaling, followed by treating the cells with LiCl for 12 hours and culture in differentiation medium for 48 hours to induce myogenic differentiation. The cell morphology was examined by immunofluorescent staining of MyHC-positive myotubes and nuclei. The differentiation and fusion indices were determined and compared among cells after the appropriate treatments. As shown in Figure 20A, the increased myotube formation in LiCl-treated cells was largely prevented in β -catenin RNAi treated cells as both differentiation and fusion of myoblasts were significantly abrogated compared with LiCl treatment alone group. DKK-1 treatment has the similar antagonistic effect on LiCl treatment as β -catenin RNAi, although to lesser extent as compared with the change that was caused by β -catenin RNAi. Furthermore, both β -catenin RNAi and DKK-1 treatment significantly blocked the expression of the fibrotic marker, ER-TR7, which was induced by LiCl treatment (Figure 20B). These results provide convincing evidence that the myogenic and fibrotic induction by LiCl treatment is mediated by β -catenin/Wnt signaling.

Forced expression of phosphorylation-resistant mutated β -catenin partially rescued the blunting effect of M-cadherin RNAi on the LiCl-induced myogenic phenotype. To further clarify the role of the phosphorylation status of β -catenin N-terminus in regulating myogenesis, we transfected a phosphorylation-resistant mutated β -catenin (S33Y- β -catenin) plasmid in C2C12 myoblasts and induced the myogenic differentiation for 48 hours after the transfection. Forced expression of S33Y- β -catenin in C2C12 myoblasts induced a significant increase in TCF/LEF transcription activity (Figure 21A) but failed to increase the protein abundance of Troponin T as determined by immunoblotting (Figure 21B), indicating that the expression of the phosphorylation-

resistant mutant form of β -catenin alone has no effect on myogenic differentiation of C2C12 cells. In contrast, LiCl treatment in cells that were co-transfected with both S33Y- β -catenin plasmid and M-cadherin siRNA, yielded an increased protein abundance of Troponin T compared with cells that were transfected with M-cadherin siRNA alone (Figure 21C). This suggests that the phosphorylation-resistant mutant form of β -catenin attenuates the blunting effect of M-cadherin RNAi on myogenic induction by LiCl treatment.

Forced expression of phosphorylation-resistant (mutated) β -catenin partially attenuated apoptosis exacerbated by M-cadherin RNAi during myogenic differentiation. We have shown previously that M-cadherin RNAi exacerbated apoptosis in myoblasts during myogenic differentiation (24). We examined this further in an attempt to determine if the phosphorylation status of β -catenin N-terminus plays a role in regulating the apoptotic phenotype caused by M-cadherin RNAi. M-cadherin-targeted siRNA and S33Y- β -catenin plasmids were either transfected alone or co-transfected into C2C12 myoblasts followed by culturing the cells in differentiation medium for 48 hours to induce myogenic differentiation. At the end of treatments, the cells were fixed and stained with an anti-MyHC antibody. *In situ* apoptotic DNA fragmentation was examined after staining the cells with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and the nuclei were counter-stained with 4',6-diamidino-2-phenylindole (DAPI) (Figure 22A). The indices for differentiation and apoptosis were calculated and compared among cells with different treatments. As shown in Figure 22, forced expression of S33Y- β -catenin alone did not alter the myogenic or the apoptotic outcomes in C2C12 cells at the end of 48-hour myogenic

differentiation. However, co-transfection of S33Y- β -catenin with M-cadherin siRNA partially rescued the impaired myogenic differentiation (Figure 22B) and attenuated the exacerbated apoptosis caused by M-cadherin RNAi alone (Figure 22C). These results suggest that alteration of the phosphorylation status of β -catenin N-terminus may mediate the apoptotic phenotype induced by disruption of M-cadherin-mediated signaling.

DISCUSSION

This is the first study to show that by modulating N-terminal phosphorylation status of β -catenin, M-cadherin-mediated signaling regulates β -catenin/Wnt signaling and promotes myogenic differentiation in a TCF/LEF transcription-independent manner. In this study we report that M-cadherin RNAi increased GSK-3 β -dependent phosphorylation of β -catenin N-terminus, decreased the protein abundance of unphosphorylated signaling-active β -catenin, and blunted myogenic induction caused by LiCl or Wnt-3a treatment. Forced expression of phosphorylation-resistant mutated β -catenin alone failed to enhance myogenic differentiation but partially rescued the myogenic and apoptotic phenotype caused by M-cadherin RNAi. Interestingly, these effects are independent of changes in TCF/LEF transcription activity.

The paired phosphorylation of β -catenin at NH₂ terminus by CK1a and GSK-3 β plays a crucial role in regulating β -catenin stability and signaling. β -catenin that is phosphorylated at residues 33 and 37 is recognized by the β -TrCP E3-ligase complex, resulting in its ubiquitylation and rapid degradation by the 26S proteasome (37). This is an important mechanism to turn off Wnt signaling. An accumulated body of evidence

suggests that N-terminus phosphorylation may directly modulate β -catenin-mediated signaling. It has been demonstrated that Wnt signaling is specifically mediated by N-terminal unphosphorylated β -catenin (38) and these N-terminal unphosphorylated β -catenin more readily accumulates in nuclei (39; 40). Thus the GSK-3 β -dependent phosphorylation of β -catenin at N-Terminus may not only induce degradation as suggested by the classical view, but may also directly impact β -catenin transcriptional activity. Thus it is possible that the phosphorylation status alone may regulate β -catenin-mediated Wnt signaling without affecting its stability. Indeed, we demonstrated in this study that the unphosphorylated active β -catenin (ABC) mainly accumulates in cytoplasm regions and not at cell-cell contacts in confluent myoblasts. In response to LiCl treatment, ABC but not total β -catenin translocates into nuclei, indicating that ABC is more responsive to Wnt stimulation.

It is clear that cadherin proteins modulate β -catenin transcription activity in a variety of cells. For example, binding of β -catenin to the cadherin cytoplasmic tail has been shown to inhibit β -catenin degradation and efficiently block its transactivation in Chinese hamster ovary and SW480 colon carcinoma cell lines (41). In addition, transient expression of exogenous E-cadherin in both epithelial and fibroblastoid cells has been reported to arrest cell growth via inhibiting β -catenin transcriptional activity (42). Furthermore, knockdown of E-cadherin in DLD-1 colorectal cancer cells, which have constitutive activation of Wnt signaling and exhibit E-cadherin-mediated cell adhesion, resulted in translocation of β -catenin to the nucleus and an enhancement of β -catenin/TCF-dependent reporter activity (43). In L929 fibroblasts, which are deficient in Wnt signaling and E-cadherin-based cell contacts, ectopic expression of E-cadherin

induced the stabilization of β -catenin at the cell junctions and caused marked alterations in cellular morphology and phenotype but no significant change in the transcriptional program of these cells in the absence of Wnt signaling. Furthermore, expression of wild-type E-cadherin significantly inhibits the growth of SW480 colon carcinoma cells via inhibiting β -catenin/TCF gene promoter activity in an adhesion-independent manner (44), whereas, that restoration of E-cadherin expression in these cells enhances the phosphorylation status of β -catenin N-terminus and suppresses the Wnt signaling in the cells. Similar to our observation in myoblasts, several other labs have demonstrated that β -catenin translocates to cell-cell junctions and this increase occurs during the initiation of myogenic differentiation (45). In addition, expression of a short 23-amino-acid segment of the cadherin cytoplasmic tail in mammalian cells does not affect cell-cell junctions but effectively inhibits β -catenin-mediated signaling (46). As RhoA activity is required for β -catenin recruitment to intercellular adhesions sites (47), it is important to note that N-cadherin-dependent cell-cell contact activates muscle-specific promoters and RhoA in C2C12 cells. To this point, no study has evaluated how M-cadherin regulates the phosphorylation status of the N-terminus of β -catenin, nor examined the impact of this regulation on myogenic differentiation. Our results in the current study show that inhibition of M-cadherin expression by RNAi increases phosphorylation of the N-terminus of β -catenin, decreases the protein abundance of unphosphorylated active β -catenin, and impairs the myogenic induction that is caused by either LiCl or Wnt-3a treatment. Together these results indicate that M-cadherin-mediated signaling attenuates GSK-3 β -dependent phosphorylation of the β -catenin N-terminus and helps in maintaining a cytosolic pool of unphosphorylated signaling-active β -catenin, which plays

a critical role in myogenic induction by Wnt stimulation. Interestingly, the positive role of M-cadherin on N-terminal unphosphorylated active β -catenin and Wnt signaling in myoblasts that is demonstrated in our study is different from the findings from another study that reported a negative regulatory role of restored E-cadherin expression on ABC and Wnt signals in colon carcinoma cells. A possible explanation for this discrepancy is that the regulatory role of cadherin on β -catenin phosphorylation is cadherin-type and cell-context dependent. The exact mechanism by which M-cadherin-mediated signaling attenuates phosphorylation of β -catenin at the N-terminus remains to be elucidated. We have shown previously that M-cadherin suppresses GSK-3 β activity in confluent myoblasts (24). In the present study we further demonstrate that while a large portion of β -catenin is detected at cell-cell contact membrane regions, in confluent myoblasts, unphosphorylated active β -catenin is located mainly in cytoplasmic regions of the cell rather than at cell-cell contacts. This observation suggests that the phosphorylation regulatory effect by M-cadherin is not simply a result of direct binding to unphosphorylated active β -catenin, but rather in an adhesion-independent manner. Furthermore, M-cadherin RNAi increases phosphorylation of the β -catenin N-terminus and decreases the protein abundance of unphosphorylated active β -catenin. Inhibition of GSK-3 β by LiCl treatment significantly reverses this effect. These results indicate that M-cadherin-mediated signaling attenuates phosphorylation of β -catenin N-terminus through suppressing GSK-3 β activity.

Our results show that the myogenic potential is much higher in confluent myoblasts as compared with cells with a low density, but the TCF/LEF transcription activities change in the opposite way. In addition, although knockdown of M-cadherin by

RNAi promotes TCF/LEF transcription activity at both steady state and LiCl/Wnt-3a-treatment conditions, it blunts the myogenic differentiation under those conditions. While forced expression of the phosphorylation-resistant mutant form of β -catenin induced a substantial increase in TCF/LEF transcription activity, it failed to induce a similar increase in myogenic differentiation by itself. These results show that there is a disconnection between the level of TCF/LEF transcription activity and the outcome of myogenic differentiation in myoblasts, suggesting that β -catenin may mediate Wnt signaling and promote myogenic differentiation independent of its role in enhancing TCF/LEF transcription activity. These findings are however, consistent with reports from treated LIM2537 cells, a poorly-differentiated colon cancer cell line, which when they are treated with the potent differentiating agent sodium butyrate, reduces GSK-3 β activity by 34%, stabilizes the level of cytoplasmic β -catenin but there is no increase in β -catenin/TCF target genes c-myc and cyclin D1 (48). Previous work has shown that β -catenin interacts with I-mfa (an inhibitor of the MyoD family of muscle regulatory transcription factors (MRFs)) and this interaction relieved the transcription activity suppression and cytosolic sequestration of MRFs by I-mfa (11). This observation suggests that a β -catenin-dependent but TCF/LEF-independent regulatory mechanism impacts MRFs during myogenesis. Similarly, Kim and colleagues (49) reported that TCF/LEF activity is dispensable for β -catenin's role in promoting myogenic differentiation. Instead, β -catenin directly interacts with MyoD and enhances its transcriptional activity that is necessary for myogenic differentiation (49). Interestingly, the results in our present study show that TCF/LEF transcription activity is increased in response to M-cadherin RNAi in spite of the decreased availability of unphosphorylated

active β -catenin, suggesting that TCF/LEF transcription activity might be more responsive to the relief of mechanical sequestration of β -catenin by M-cadherin caused by M-cadherin knockdown and less dependent on the change in phosphorylation status of β -catenin N-terminus, at least in C2C12 myoblasts.

Along with Notch signaling, the β -catenin/Wnt signaling pathway plays a critical role in regulating myogenic differentiation (34; 35; 50; 51). Although skeletal muscle progenitor cells are committed to be myogenic lineages and do not spontaneously adopt non-myogenic fates (52), it has been reported that hyper-activation of Wnt signaling may induce the premature termination of the expansion of myogenic progenitor cells and push the myogenic progenitor cells towards fibroblast lineages, resulting in the increased fibrosis in muscle tissues (34; 35). Indeed, we demonstrated that treating C2C12 myoblasts with LiCl before culturing them in differentiation medium gave rise to the expression of a fibroblast marker, ER-TR7. The fibrotic induction caused by Wnt stimulation via LiCl treatment could be effectively reversed by inhibition of the expression of either M-cadherin or β -catenin.

We have shown previously that knockdown of M-cadherin expression via RNAi in both C2C12 myoblasts and primary muscle progenitor cells exacerbates apoptosis during myogenic differentiation. This increase in apoptotic signaling was partially rescued by inhibition of GSK-3 β activity (24). In the current study, we further demonstrated that M-cadherin RNAi increased phosphorylation of β -catenin N-terminus and forced expression of the phosphorylation-resistant mutated β -catenin partially rescued the apoptosis induced by M-cadherin RNAi. These observations suggest that alteration in the phosphorylation levels of β -catenin N-terminus and β -catenin/Wnt

signaling might be at least partially responsible for mediating M-cadherin RNAi-induced apoptosis during myogenic differentiation. Our findings here are consistent with reports from other groups that show that β -catenin/Wnt signaling plays an important role in promoting survival of either skeletal or cardiac myoblasts under various conditions (53-56).

In conclusion, the data in the present study show that M-cadherin-mediated signaling attenuates phosphorylation of β -catenin N-terminus and helps in maintaining a cytosolic pool of N-terminal unphosphorylated signaling-active β -catenin which plays a key role in mediating canonical Wnt signaling to promote myogenic differentiation in a TCF/LEF-independent manner.

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The authors have declared that no competing interests exist.

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Figure 17

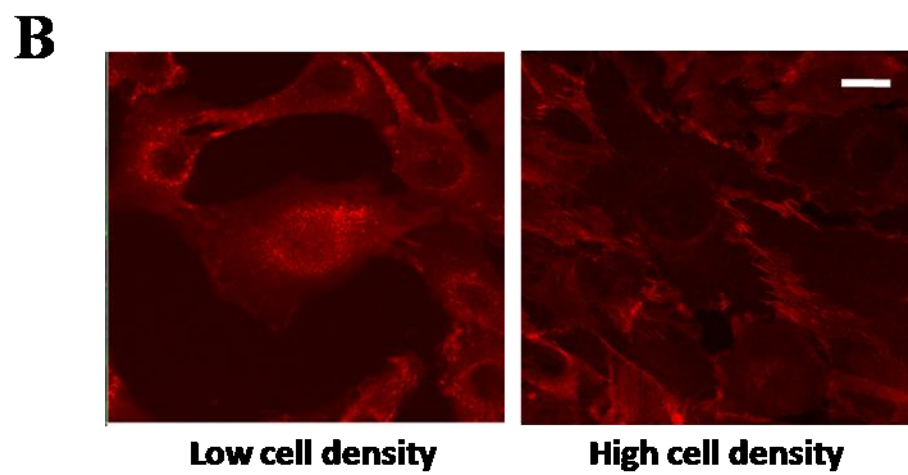
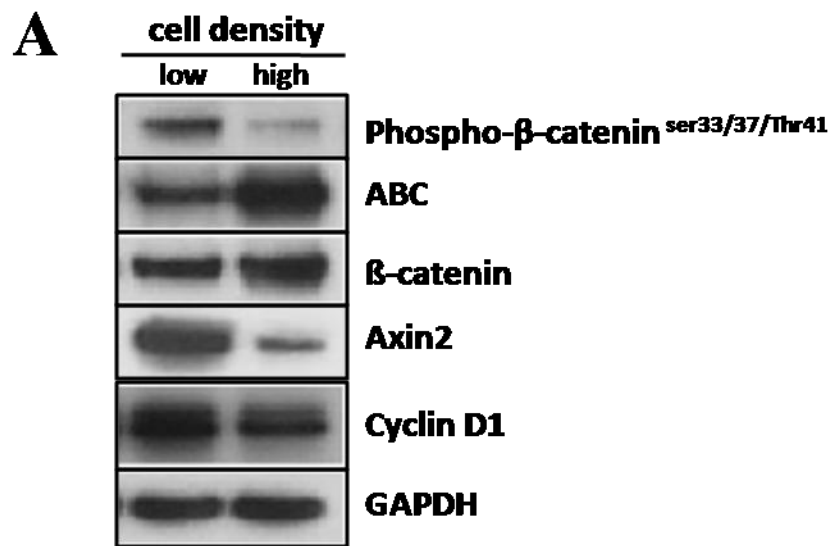


Figure 17

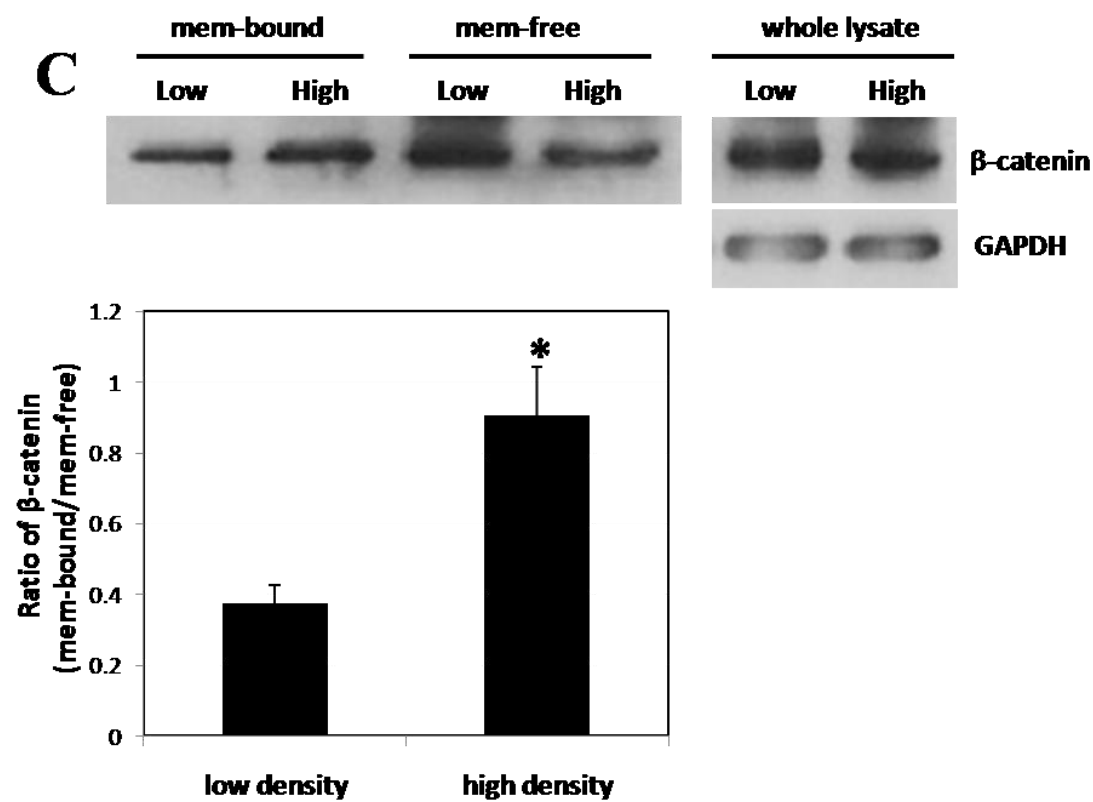


Figure 17

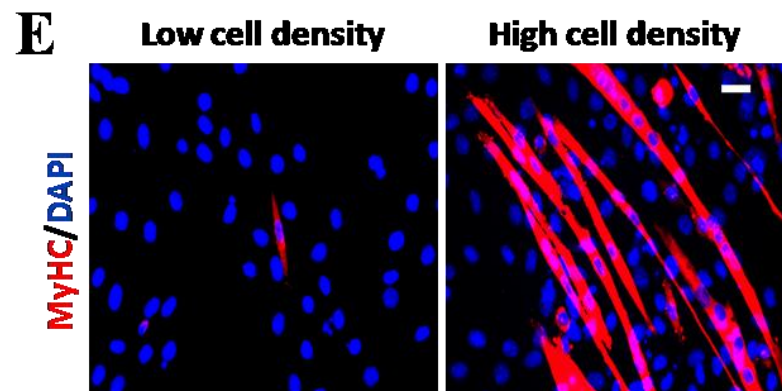
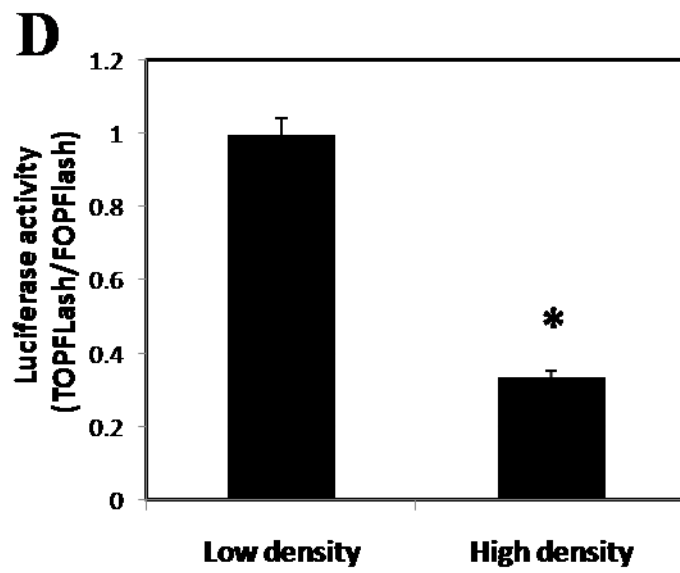


Figure 17. Phosphorylation and subcellular distribution of β -catenin, TCF/LEF transcription activity, and myogenic differentiation in C2C12 myoblasts at different cell densities.

A. C2C12 myoblasts were plated at either a low density ($2.0 \times 10^3/\text{cm}^2$), or a high density ($2.1 \times 10^4/\text{cm}^2$). 48 hours after plating, the cells were harvested in lysis buffer for immunoblotting analysis of the protein abundance of phosphorylated (Ser33/37/Thr41), Ser37/Thr41-unphosphorylated active β -catenin (ABC) and total β -catenin. Protein abundance of Axin2 and cyclin D1 were also examined. GAPDH was used as a loading control.

B. Representative confocal images of β -catenin staining in C2C12 myoblasts at low or high cell density as described in Figure 1A. The objective magnification is 63x. The length of scale bar is 10 μm .

C. Top panel: Representative immunoblots of protein abundance of β -catenin in subcellular fractions (membrane-bound versus membrane-free) of cells at low or high density as determined by cell surface biotinylation assay. Bottom panel: determination and comparison of ratios of β -catenin in membrane-bound fraction to that in membrane-free fraction in cells of different densities. Each data point (mean \pm SEM) is the mean of three independent experiments. *, $P < 0.05$ vs. the low density group.

D. C2C12 myoblasts transfected with either a TOPFlash or a FOPFlash plasmid. The cells were then plated at low or high cell density. 48 hours later the cells were harvested for luciferase activity measurement to determine the TCF/LEF transcription activity.

Each data point (mean \pm SEM) is the mean of three independent experiments. *, $P < 0.05$ vs. the low density group.

E. Representative images of MyHC-positive myotubes in cells at low or high density after culture in differentiation medium for 48 hours. MyHC: red; DAPI: blue. Images were acquired at 20x. The scale bar = 100 μm .

Figure 18

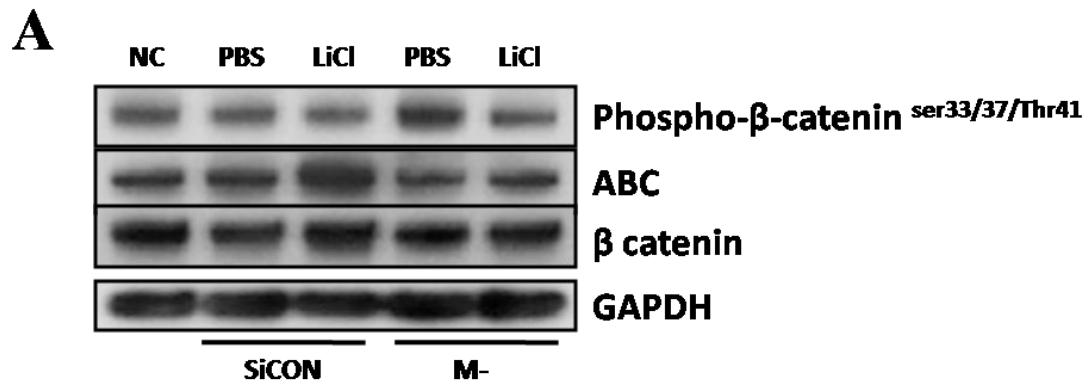


Figure 18

B

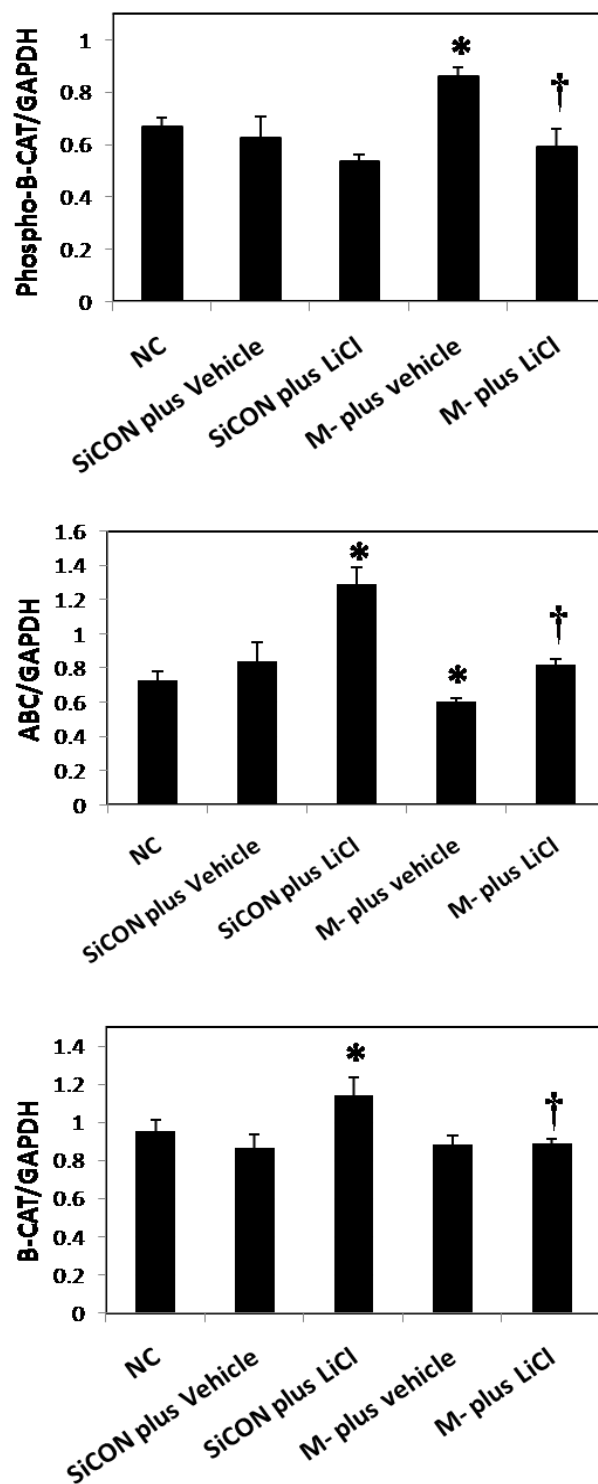
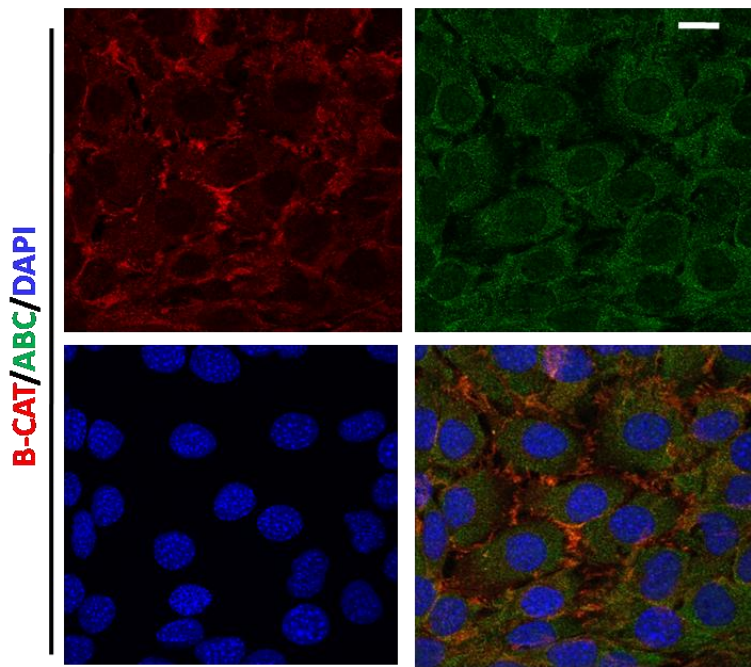


Figure 18C

i, SiCON plus PBS



ii, SiCON plus LiCl

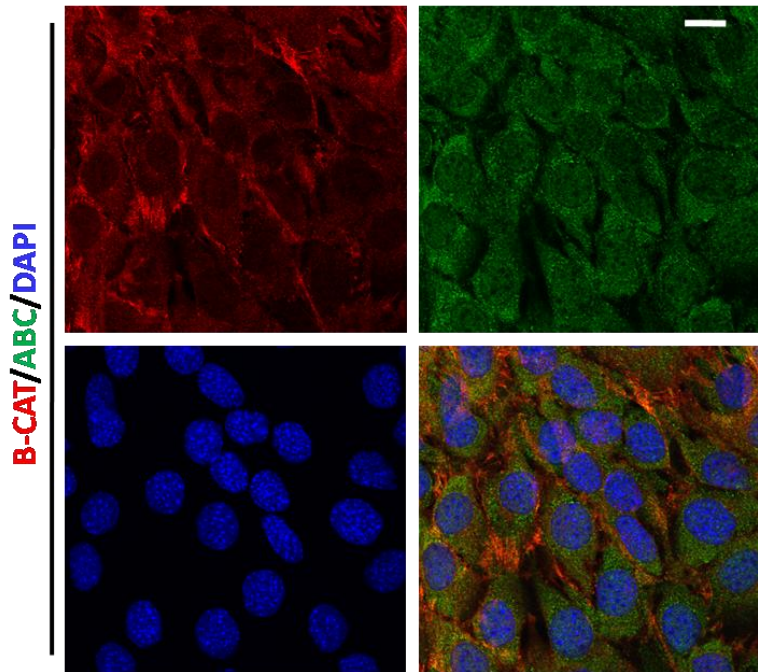
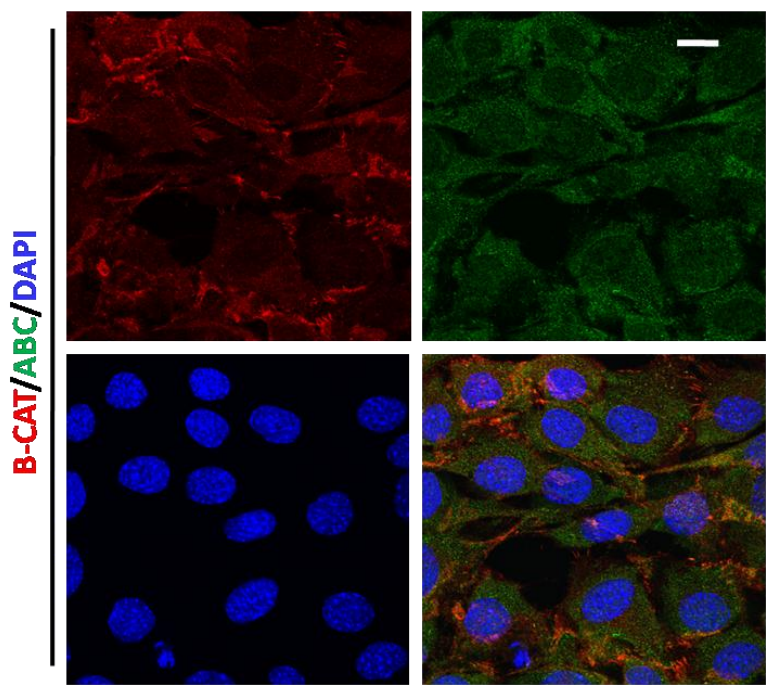


Figure 18C

iii, M- plus PBS



iv, M- plus LiCl

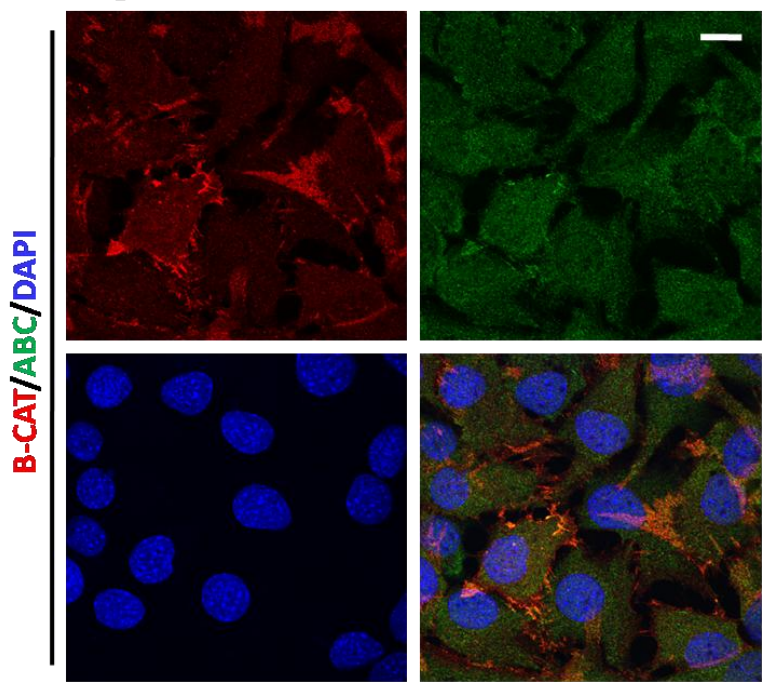


Figure 18

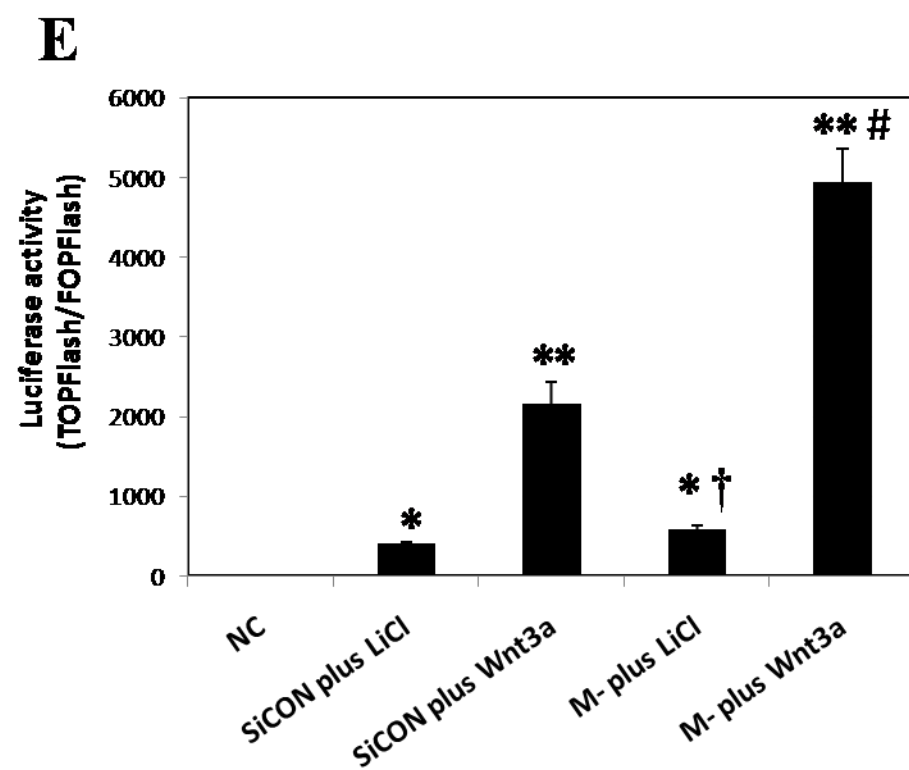
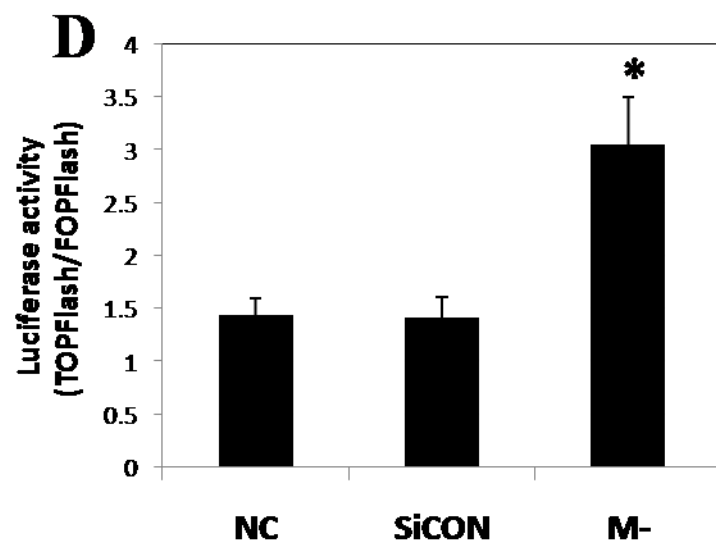


Figure 18. Effect of M-cadherin RNAi and LiCl treatment on β -catenin phosphorylation and subcellular distribution, and TCF/LEF transcription activity.

A. Representative immunoblots of protein abundance of phospho-, unphosphorylated active β -catenin (ABC) and total β -catenin in C2C12 myoblasts in either M-cadherin siRNA-transfected (M-) or non-targeted scrambled siRNA-transfected (SiCON) cells treated with LiCl (20mM) or PBS as a vehicle control for 12 hours. Non-treated cells with same culture condition were used as a normal control (NC). GAPDH was probed as a loading control.

B. Top panel: Densitometric analyses of immunoblots of phospho- β -catenin normalized to GAPDH. *, $P < 0.05$ vs. the SiCON plus Vehicle group. †, $P < 0.05$ vs. M- plus vehicle group. Middle panel: Densitometric analyses of immunoblots of ABC normalized to GAPDH. *, $P < 0.05$ vs. the SiCON plus Vehicle group. †, $P < 0.05$ vs. M- plus vehicle group. Bottom panel: Densitometric analyses of immunoblots of total β -catenin normalized to GAPDH. *, $P < 0.05$ vs. the SiCON plus Vehicle group. †, $P < 0.05$ vs. SiCON plus LiCl group.

C. Representative confocal images of immunofluorescent staining of ABC and total β -catenin in M-cadherin-RNAi-treated (M-) or non-targeted scrambled siRNA-transfected (SiCON) cells that were treated with PBS or LiCl for 12 hours. ABC: green; β -catenin: red; DAPI: blue. Images were acquired at 63x. The length of scale bar is 10 μ m.

D. TCF/LEF transcription activity in M-cadherin-RNAi (M-), non-targeted scrambled siRNA-transfected (SiCON) or normal control (NC) C2C12 myoblasts as determined by TOPFLASH/FOPFLASH luciferase activity. *, $P < 0.05$ vs. the control groups.

E. TCF/LEF transcription activity in M-cadherin-RNAi (M-), non-targeted scrambled siRNA-transfected (SiCON) or normal control (NC) C2C12 myoblasts that were treated with LiCl (20mM) or recombinant Wnt-3a (50ng/ml) for 12 hours as determined by TOPFLASH/FOPFLASH luciferase activity. *, $P < 0.05$ vs. the normal control (NC) group. **, $P < 0.01$ vs. NC group. †, $P < 0.05$ vs. the SiCON plus LiCl treatment group. #, $P < 0.05$ vs. the SiCON plus Wnt-3a treatment group.

Figure 19

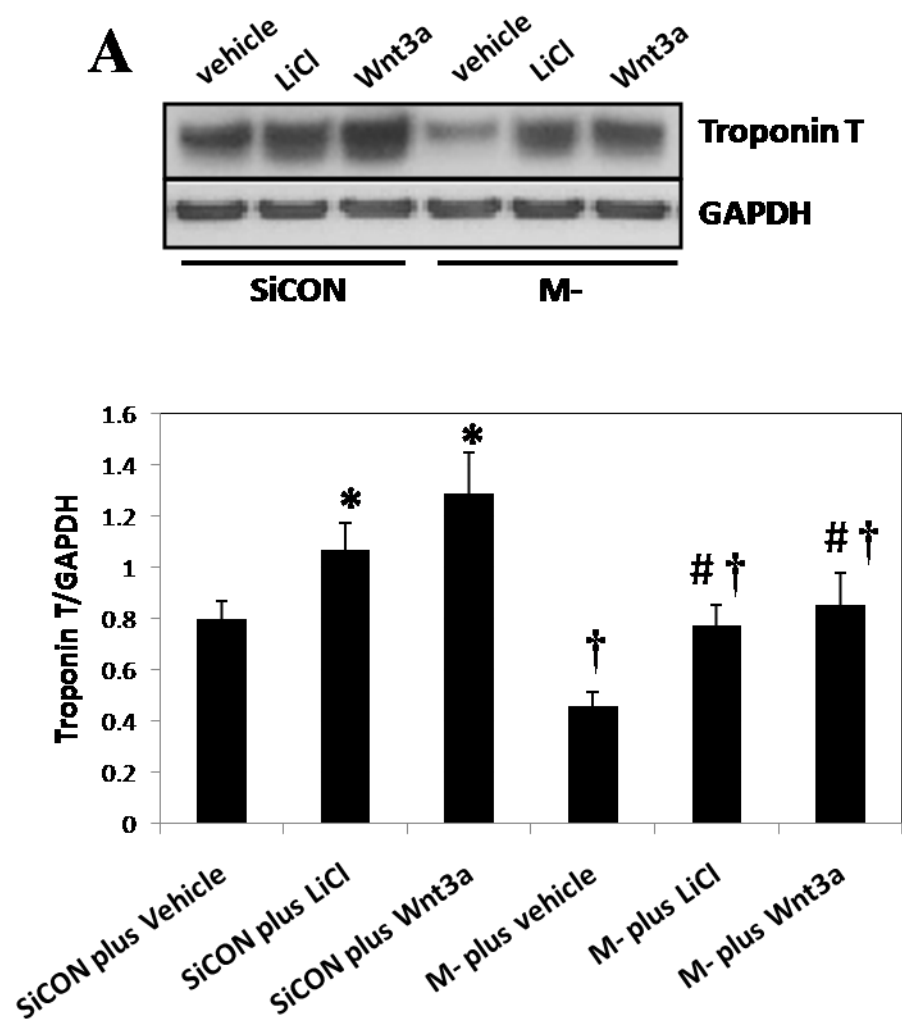


Figure 19

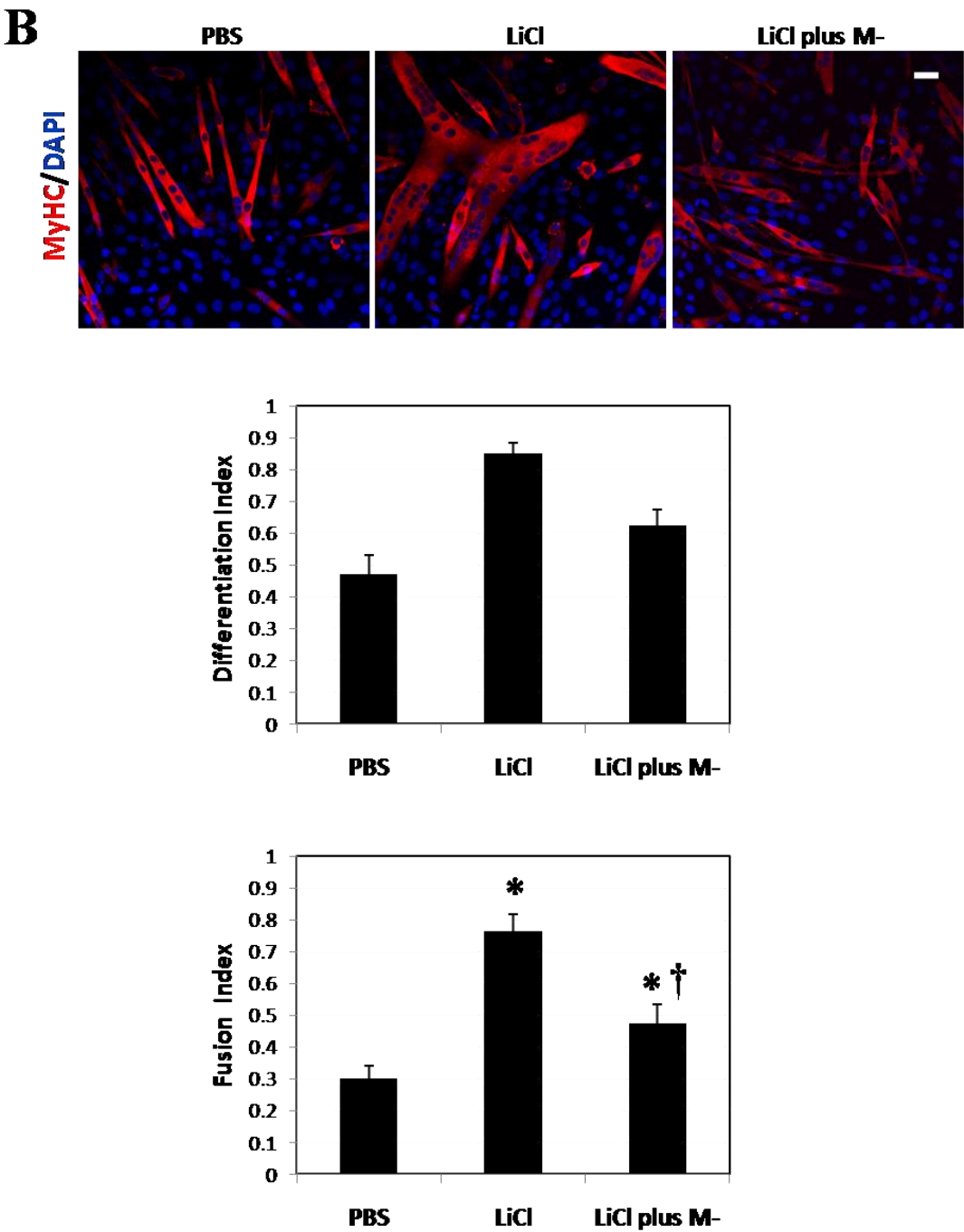


Figure 19

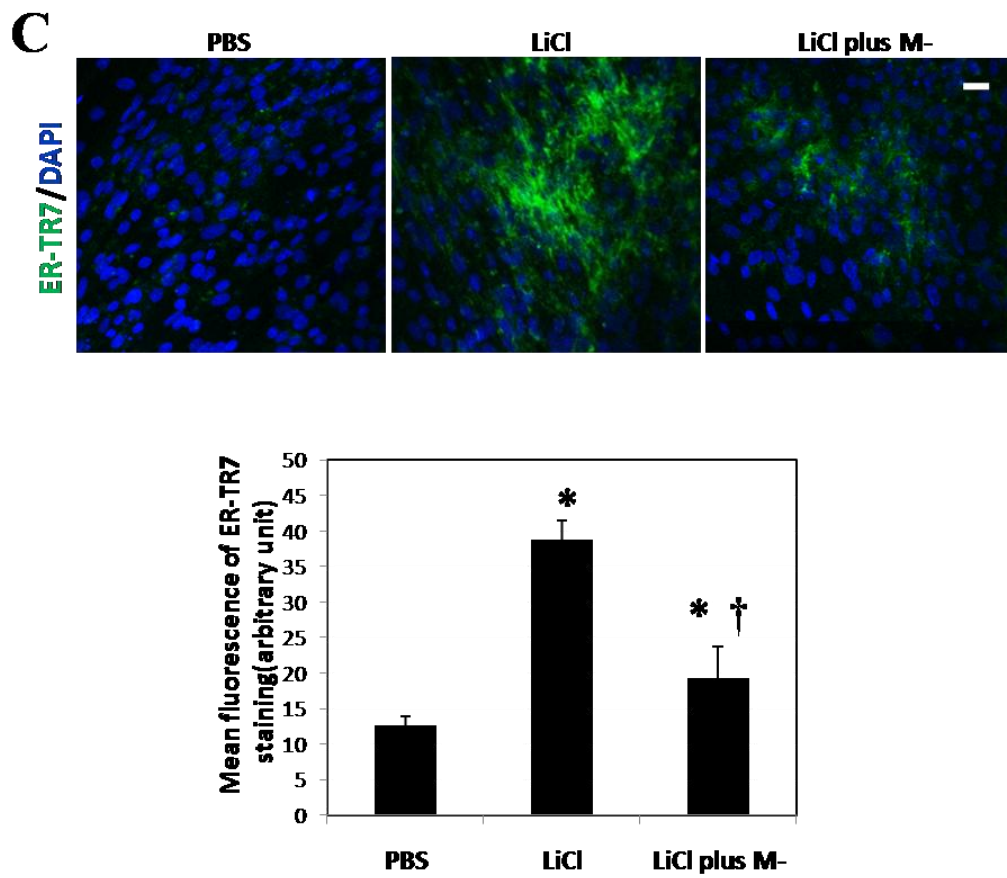


Figure 19. Effect of M-cadherin RNAi and LiCl treatment on myogenic and fibrotic outcome in C2C12 myoblasts.

A, Top panel: Representative immunoblots of protein abundance of Troponin T, an established marker for terminal myogenic differentiation, in M-cadherin-RNAi-treated (M-) or non-targeted scrambled siRNA-transfected (SiCON) cells that were treated with LiCl (20mM), recombinant Wnt-3a (50ng/ml), or DMSO as a vehicle control for 12 hours, followed by culture in differentiation medium for 48 hours. GAPDH was probed as a loading control. **Bottom panel:** Densitometric analysis of immunoblots of Troponin T normalized to GAPDH. *, $P < 0.05$ vs. the SiCON plus Vehicle group. #, $P < 0.05$ vs. M- plus Vehicle group. †, $P < 0.05$ vs. the correspondent vehicle/LiCl/Wnt-3a-treatment in SiCON groups.

B, Top panel: Representative confocal images of MyHC-positive C2C12 myotubes after treatments with PBS, or LiCl, or LiCl plus M-cadherin RNAi (LiCl plus M-), followed by culture in differentiation medium for 48 hours. MyHC: red; DAPI: blue. Images were acquired at 20x. The length of scale bar is 100 μ m. **Middle panel:** The differentiation index was determined and compared among cells with above treatments. **Bottom panel:** The fusion index was determined and compared among cells with above treatments. *, $P < 0.05$ vs. the PBS-treated group. †, $P < 0.05$ vs. the LiCl-treated group.

C. Top panel: Representative confocal images of immunofluorescent staining of a fibrotic marker, ER-TR7, in C2C12 myoblasts with treatments as described in figure 3B. ER-TR7: green; DAPI: blue. Images were acquired at 20x. The length of scale bar is 100 μ m. **Bottom panel:** Quantitative analysis of mean fluorescence intensity of ER-TR7

staining in cells with above treatments. *, $P < 0.05$ vs. the PBS-treated group. †, $P < 0.05$ vs. the LiCl-treated group.

Figure 20

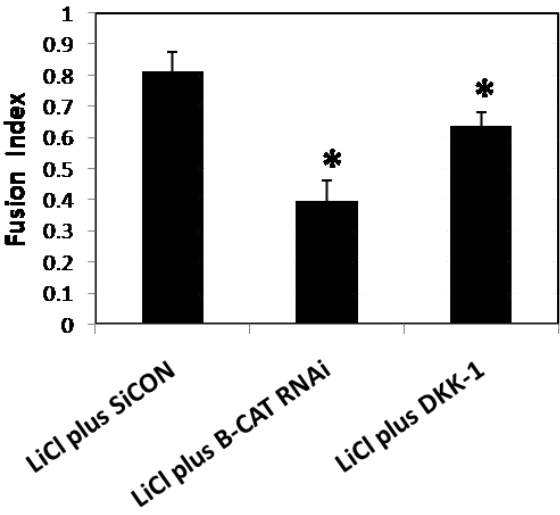
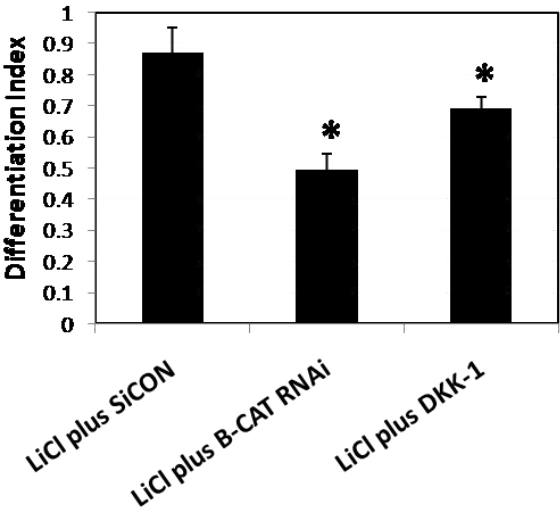
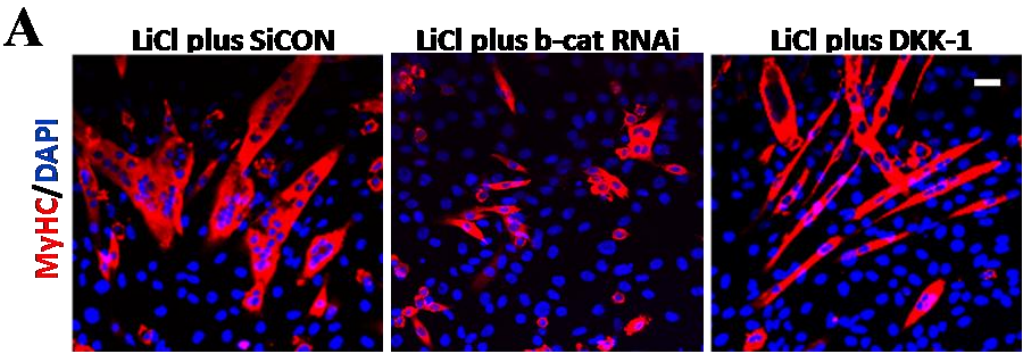


Figure 20

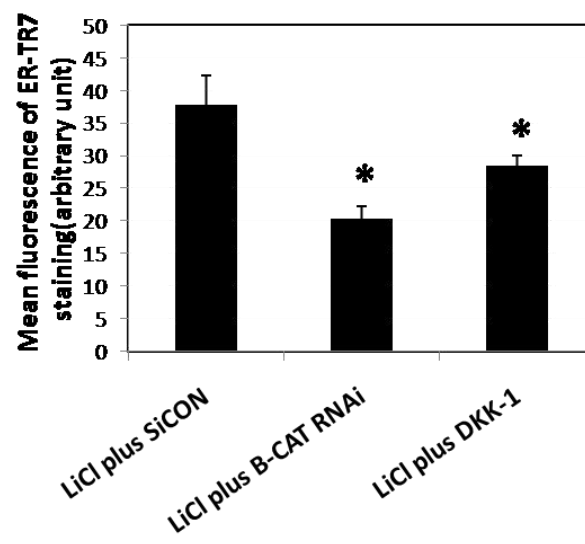
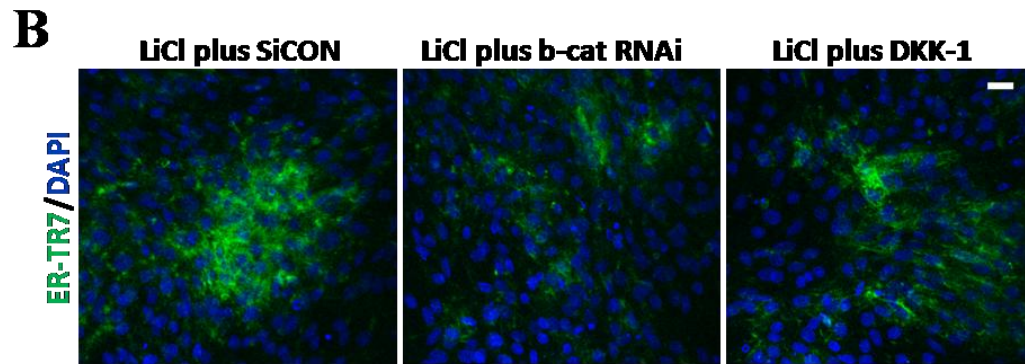


Figure 20. The effect of β -catenin RNAi and DKK-1 treatment on myogenic and fibrotic induction by LiCl treatment.

A. Top panel: Representative confocal images of MyHC-positive C2C12 myotubes after treatments of β -catenin RNAi, or SiCON, or DKK-1(200ng/ml), followed by LiCl treatment for 12 hours and culture in differentiation medium for 48 hours. MyHC: red; DAPI: blue. Images were acquired at 20x. The length of scale bar is 100 μ m. **Middle panel:** Differentiation index among cells with above treatments. **Bottom panel:** Fusion index among cells with above treatments. *, $P < 0.05$ vs. the LiCl plus SiCON group.

B. Top panel: Representative confocal images of immunofluorescent staining of ER-TR7 in C2C12 myoblasts with treatments as described in Figure 5A. ER-TR7: green; DAPI: blue. Images were acquired at 20x. The length of scale bar is 100 μ m. **Bottom panel:** Quantitative analysis of mean fluorescence intensity of ER-TR7 staining in cells with above treatments. *, $P < 0.05$ vs. the LiCl plus SiCON group.

Figure 21

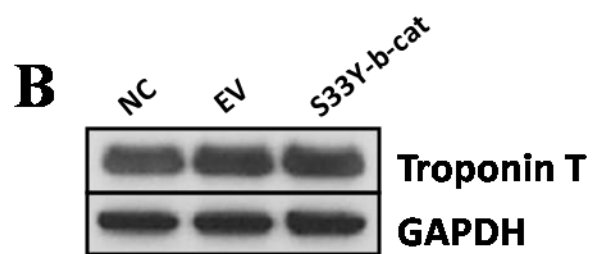
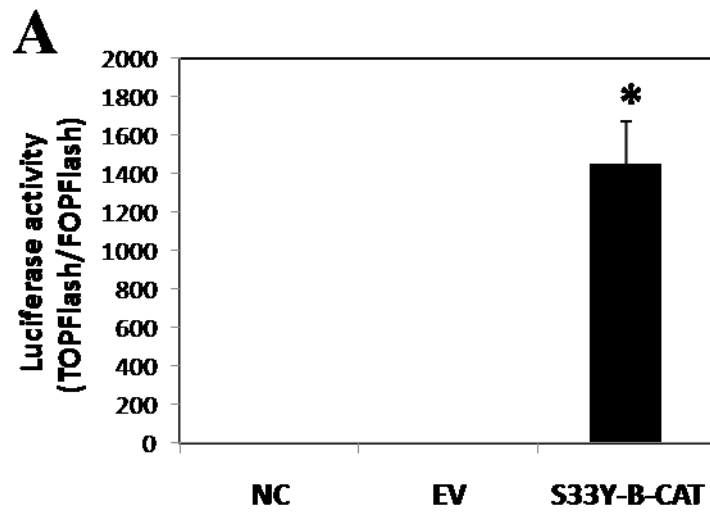


Figure 21

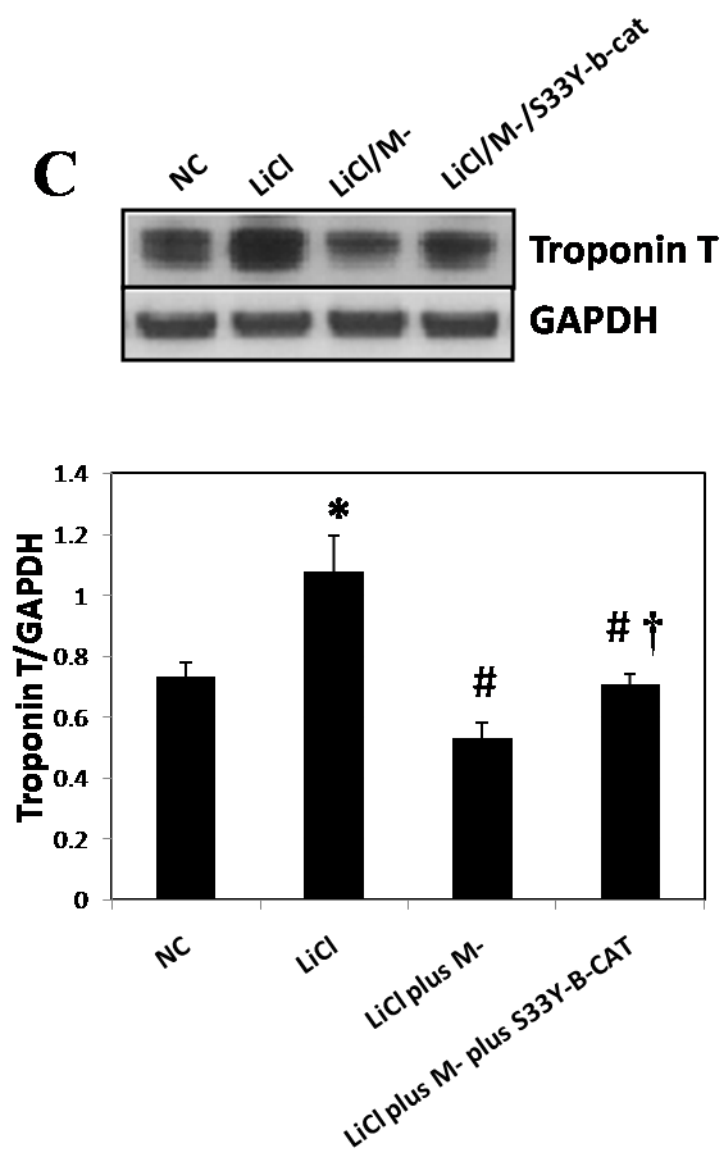


Figure 21. The effect of forced expression of phosphorylation-resistant mutated S33Y- β -catenin on TCF/LEF transcription activity and myogenic differentiation.

A. TCF/LEF transcription activity in S33Y- β -catenin plasmid-transfected (S33Y-B-CAT), empty vector-transfected (EV) or untreated normal control (NC) C2C12 myoblasts was determined by measuring TOPFLASH/FOPFLASH luciferase activity. *, $P < 0.01$ vs. the control groups.

B. Representative immunoblots of protein abundance of Troponin T in S33Y- β -catenin-transfected (S33Y-B-CAT), empty vector-transfected (EV) or normal control (NC) C2C12 myoblasts after culture in differentiation medium for 48 hours. GAPDH was probed as a loading control.

C. Top panel: Representative immunoblots of protein abundance of Troponin T in the following four groups of cells: normal control (NC), LiCl-treated (LiCl), LiCl plus M-cadherin siRNA-transfected (LiCl/M-), LiCl plus M-cadherin-siRNA and S33Y- β -catenin plasmid-co-transfected (LiCl/M-/S33Y-B-CAT), and differentiation for 48 hours. **Bottom panel:** Densitometric analyses of immunoblots of Troponin T normalized to GAPDH. *, $P < 0.05$ vs. NC group. #, $P < 0.05$ vs. LiCl group. †, $P < 0.05$ vs. LiCl plus M- group.

Figure 22

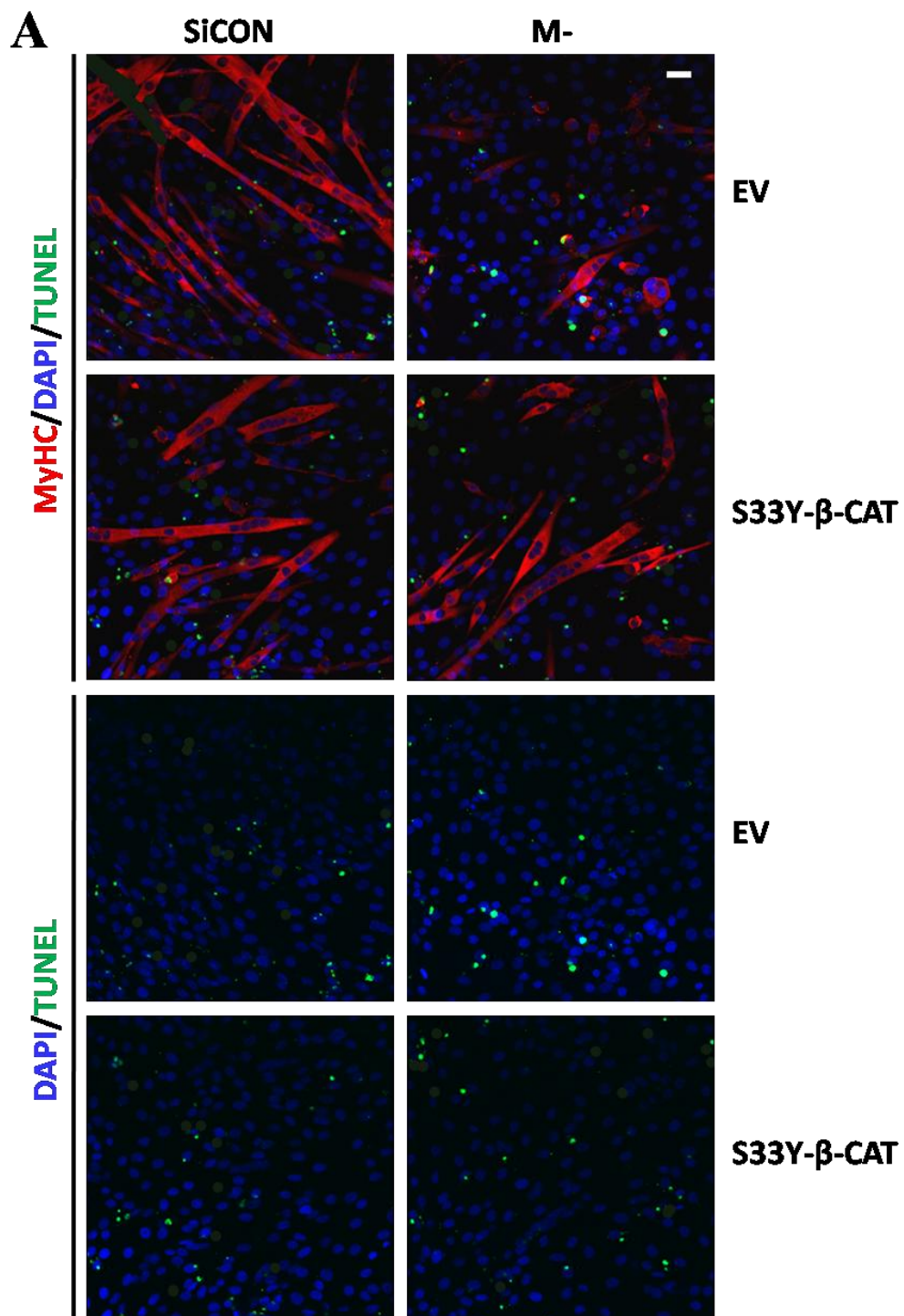
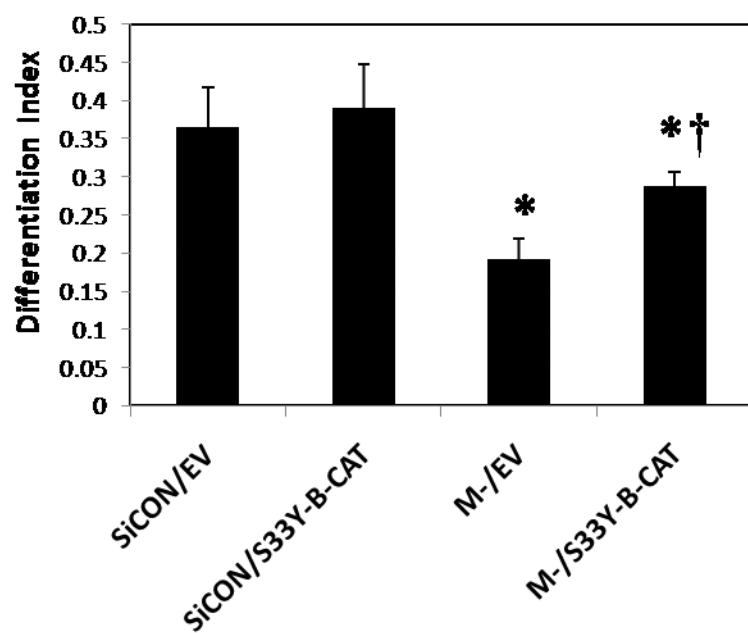


Figure 22

B



C

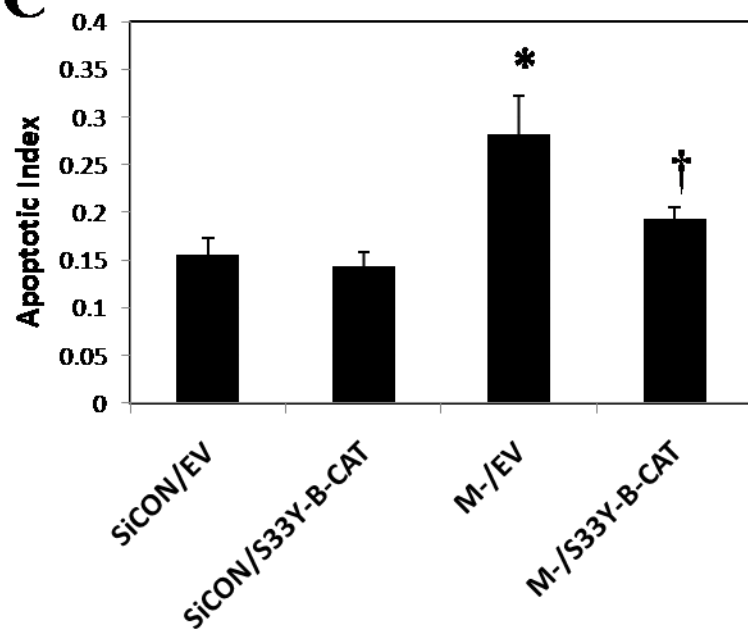


Figure 22. Effect of forced expression of phosphorylation-resistant mutated S33Y- β -catenin on apoptosis and myogenic differentiation in response to M-cadherin RNAi.

C2C12 cells were grown on coverslips, co-transfected with M-cadherin-targeted (M-) or non-targeted scrambled siRNA (SiCON) with S33Y- β -catenin plasmid (S33Y-B-CAT) or an empty vector (EV) for 36 hours. The cells were then cultured in differentiation medium for 48 hours.

A. Representative confocal images of immunofluorescent staining MyHC-positive C2C12 myotubes with treatments as described above. In situ DNA fragmentation was labeled by TUNEL staining. MyHC: red; TUNEL: green; DAPI: blue. Images were acquired at 20x. The scale bar = 100 μ m.

B. The differentiation index of C2C12 cells that were exposed to the above treatments.

*, $P < 0.05$ vs. SiCON/EV group. †, $P < 0.05$ vs. M-/EV group.

C. The fusion index of C2C12 cells exposed to the treatments described above. *, $P < 0.05$ vs. SiCON/EV group. †, $P < 0.05$ vs. M-/EV group.

CHAPTER 4:
General discussion, conclusion and future studies

4.1. General discussion and conclusion. Skeletal muscle comprises about 40% of human body weight, and plays a key role in various functions including locomotion, maintenance of posture, respiration (diaphragm and intercostals contractions), communication (by verbal and facial muscles), thermogenesis and regulation of metabolism (1). Mammalian skeletal muscle is comprised of multinucleated myofibers that are formed by fusion of mononucleated myoblasts during embryonic development. As a contractile tissue that undergoes repeated contraction, damage occurs frequently to muscles due to the wear and tear during repeated contractions, especially in response to lengthening contractions. However, adult skeletal muscle is able to regenerate and repair the injury. Thus muscle regeneration is critical for maintenance of adult muscle mass, quality and performance.

A population of muscle precursor cells known as satellite cells, function as stem cells in adult skeletal muscle and are critical for muscle regeneration. In addition, growth and regeneration of muscle as a postnatal tissue requires additional myonuclei from outside of myofibers since myonuclei are post-mitotic (2). Satellite cells serve as the primary source for the addition of new myonuclei to adult muscle tissue (3). The decrease in both number and function of satellite cells is one of the major cellular mechanisms for various muscular wasting disorders including sarcopenia. It is one of the long term research focuses in the Alway lab to get a better understanding on the mechanism of sarcopenia. Previous findings in the Alway lab have identified an association between increased apoptotic signaling and the occurrence and progression of sarcopenia (4-9). The overall purpose of this study is to extend the investigation of the association between apoptosis regulation and muscle regeneration to a cellular and

molecular level, and to get further insights in the molecular mechanism of apoptotic regulation of myoblasts and the effect of this regulation on myogenic differentiation and adult muscle regeneration. Understanding the regulatory role of M-cadherin in apoptosis and Wnt signaling was one of the primary goals of this research. Although an *in vivo* M-cadherin knockout model suggests that M-cadherin is dispensable for myogenesis and muscle regeneration (10), the extensive potential compensations that occur in a genetically engineered animal model may not fully represent the consequences caused by a gene down-regulation that occurs in an adult context. For example, MyoD and Myf5, are both myogenic regulatory factors (MRFs) that are important in regulating the behavior of satellite cells. MyoD determines the myogenic capability of myoblasts (11; 12), whereas Myf5 plays a major role in regulating their self-renewal and homeostasis (13; 14). Interestingly, although either MRF can compensate for the loss of the other during embryogenesis, they fail to compensate for each other in an adult context. The same mechanism might also apply to the interpretation of the redundancy among different types of cadherins. Although the loss of M-cadherin appears to be well compensated during embryogenesis, the same compensation by other types of cadherins may not occur as the gene expression of M-cadherin is down-regulated in adult muscle. This speculation is supported by findings from *in vitro* studies from different groups which reported that M-cadherin promotes myoblast fusion (15; 16) and is indispensable for myogenic differentiation (17). In addition, in spite of the important role of cadherin in regulating apoptosis and canonical Wnt signaling in other types of cells and tissues, there is no report that addresses how M-cadherin modulates apoptosis and Wnt signaling in myoblasts. The study presented in Chapter 2 is the first

report to show that M-cadherin protects myoblasts against apoptosis during myogenic differentiation. We demonstrated that M-cadherin-mediated cell-cell adhesion activates PI3K/Akt and enhances the inhibitory phosphorylation of GSK-3 β at serine 9, suppresses the occurrence of MOMP and protects the cells against mitochondria-associated intrinsic apoptosis during myogenic differentiation (Figure 4.1). Furthermore, as the data presented in Chapter 3, this is the first study to show that by modulating the GSK-3 β -dependent phosphorylation of β -catenin at the N-terminus, M-cadherin-mediated signaling helps in maintaining a signaling-active N-terminal unphosphorylated β -catenin, which is critical in mediating the myogenesis-promoting effect of canonical Wnt signaling in a TCF/LEF-independent manner (Figure 4.1).

Substantial evidence shows that apoptosis is a coincident event that occurs during cell differentiation in many types of cells including myoblasts (18). Indeed, a substantial portion of myoblasts die by apoptosis whereas other ones survive by differentiation and fusion into myotubes (18-22). The underlying mechanism and signaling pathways controlling the life and death decisions of differentiating cells are largely unknown and therefore the object of intensive investigation. It is known that proper mitochondria function is critical in myogenic differentiation (23-25). Furthermore, there is a difference in mitochondrial membrane potential between myoblasts undergoing apoptosis and myoblasts that successfully differentiate (26). The current study demonstrates that M-cadherin-mediated signaling plays an important role in maintaining the mitochondrial integrity of differentiating myoblasts and regulates apoptosis during myogenic differentiation. We show that inhibition of M-cadherin expression by RNAi sensitized C2C12 myoblasts to mitochondria-associated intrinsic

apoptosis that was triggered by cell confluence and serum starvation. This observation suggests that M-cadherin-mediated signaling regulates mitochondrial integrity and function in myoblasts.

Cadherin is an established inhibitor for β -catenin/canonical Wnt signaling in various types of cell contexts. It inhibits β -catenin activity by three ways: mechanical sequestration, direct inhibition of its transcription activity, and promoting its N-terminus phosphorylation by GSK-3 β (in colon cancer cells) (27). Interestingly, different from the finding in a colon cancer cell line, we found in myoblasts that M-cadherin-mediated signaling attenuates the phosphorylation of β -catenin N-terminus and promotes β -catenin activity in a TCF/LEF-independent manner. This observation adds a novel mechanism to the regulation of β -catenin by cadherins. In addition, the data in chapter 3 demonstrates that hyperactivation of Wnt signaling caused by LiCl treatment induces both myogenic differentiation and a conversion from a myogenic lineage to a fibroblastic one, which is consistent with a study from the Rando group (28). Moreover, inhibition of M-cadherin expression by RNAi reversed this effect, suggesting that M-cadherin-mediated signaling is required by both myogenic differentiation and fibroblastic conversion induced by the activation of β -catenin/canonical Wnt signaling. This brings up a question, that is, will overexpression of M-cadherin in skeletal muscle lead to muscle fibrosis, which remains to be elucidated in a future study. On the other hand, although it is assumed that muscle fibrosis is a pathological procedure which has detrimental effects on the muscle quality and function, the mutual effects between myogenic myoblasts and fibroblasts during the process of myogenesis have drawn increasing attention and generated interesting observations. Emerging evidence from

different groups have shown that in addition to its classical role in adult muscle structure and function, connective tissue is an important component of the niche where muscle progenitors reside and is a critical regulator of myogenesis (29; 30). The mutual interactions between fibroblasts and satellite cells promote an efficient, effective myogenic differentiation (29-31).

In conclusion, the data presented in this dissertation demonstrates that M-cadherin plays a critical role in regulating the fate of myoblasts by promoting their survival against mitochondria-associated intrinsic apoptosis during myogenic differentiation via PI3K/Akt/GSK-3 β pathway. Furthermore, by modulating the phosphorylation status of β -catenin N-terminus, M-cadherin regulates β -catenin/canonical Wnt signaling in a TCF/LEF-independent manner which is crucial for myogenic differentiation (Figure 23). These findings identify a novel mechanism underling the regulation of myoblast's behavior and fate during muscle regeneration.

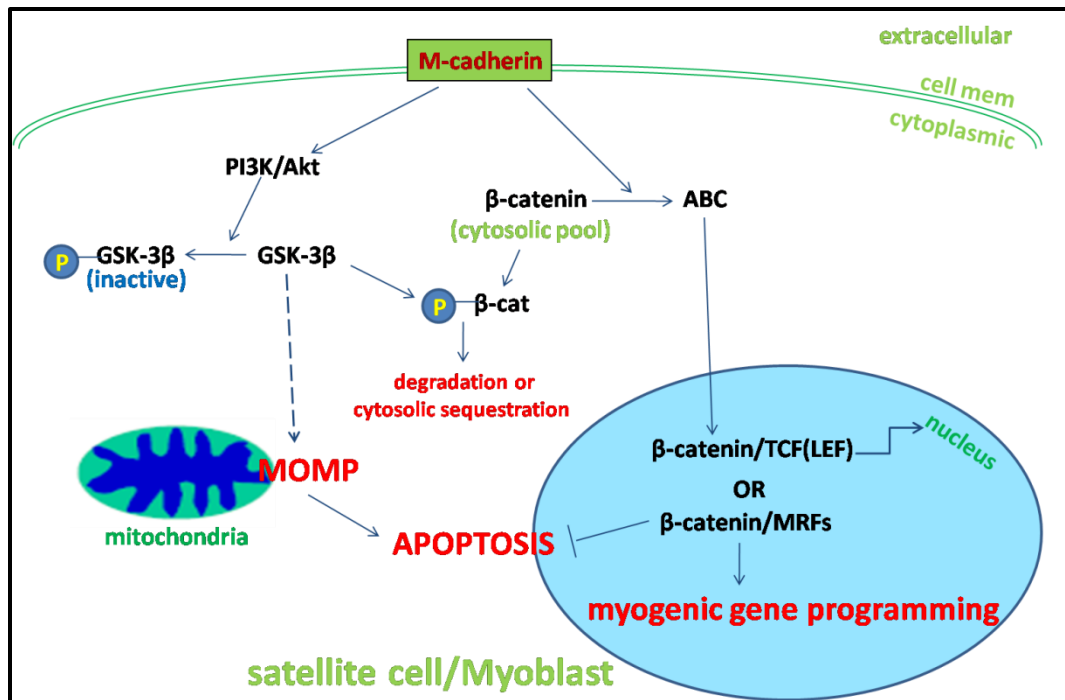


Figure 23. Schematic summary of the regulatory role of M-cadherin in myoblast's apoptosis/survival and myogenic differentiation.

"- \rightarrow " in the figure suggests that certain phosphorylation substrates of GSK-3 β that are involved in the occurrence of MOMP, such as Bax (32), are not included in this figure since they are beyond the scope of this dissertation.

4.2. Significance of the findings and practical implications for treatment of human diseases.

Decrease in both number and functionality of satellite cells is among the major contributing factors to the onset and progress of sarcopenia (33). In aged muscle, the number of M-cadherin-positive satellite cells is also lower (34). Prior to the data presented in this dissertation, it was not known whether there is a correlation between a decreased M-cadherin expression and the decline in both number and function of satellite cells in aged muscle. The data in the present study demonstrates that M-cadherin promotes myoblast's differentiation and reduces apoptotic signaling by suppressing GSK-3 β activity and enhancing β -catenin signaling activity. In contrast to

cadherin signaling outcomes in many other cell types, this observation supports a novel hypothesis that the disruption of M-cadherin-mediated cell-cell and cell-niche adhesions could be a possible mechanism that contributes to the decreased number and function of satellite cells in aged muscle, which gives rise to the onset and progression of sarcopenia and other muscle wasting disorders. The implications of the findings in this study are that gene overexpression of M-cadherin in aged muscle might be used as a potential therapeutic intervention that could rescue or at least partially attenuate the decrease in both number and functionality of satellite cells in aged muscle since enhanced M-cadherin-mediated signaling would provide signals that promote the survival and myogenic capability of satellite cells in aged muscle.

Cadherins are an important component of the stem cell niche and have been reported to play a critical role in mediating the recruitment and adhesion of stem cells to their niche (35-38). A niche to stem cells is an anatomic residence for the cells to stay in. But more importantly, it is also a functional dimension that enables the cells to reproduce, self-renew, and survive in a stressful environment (39). Data from the current project could be eventually used to support therapies for muscular dystrophy. For example, in current experimental approaches or clinical trials that muscle progenitor cells or stem cells from other lineages are transplanted into a host with muscular dystrophic disorders such as Duchene Muscular Dystrophy, the low survival and engraftment rate of donor progenitor cells after transplantation has been a major barrier to the full success of this stem-cell-based therapies (40; 41). In addition, the *in vitro* handling and expansion of the donor cells before transplantation also decrease the *in*

vivo myogenic capability of the cells dramatically after they are injected into the host muscles (42; 43). Thus, either *in vitro* culture or injection of the cells into the host sites as foreign grafts may cause the loss of the original signals from the niche that promote survival and myogenic capability of the cells because the regular biological niche becomes temporarily or permanently unavailable to the transplanted cells. This could be an important contributing factor to the low survival and engraftment rate, as well as the poor myogenic performance, of the cells after transplantation. The survival- and myogenic promoting effect of M-cadherin demonstrated in this study provides a potential novel therapeutic approach. We propose that by increasing the gene expression of M-cadherin in the donor cells and the niches in host muscles, the survival and engraftment rate of cells after transplantation in muscle tissue will be improved, and the myogenic capability of the transplanted cells will also be increased. Thus the therapeutic outcome of the stem cell-based strategies in treating muscular dystrophies will be greatly improved. More importantly, this M-cadherin-overexpression hypothesis demonstrates the crucial role of a healthy niche in maintaining the survival, self-expansion, and myogenic capability of muscle stem cells, not only for their survival and engraftment *in vivo*, but also during the process of expansion *in vitro*. The cells are isolated from the donor body and cultured *in vitro* to expand into enough number before they are ready to be injected into the host muscles. However, instead of culturing in a traditional cell culture system, an artificially modified and engineered culture system which is enriched with M-cadherin and other key adhesion molecules that are found in a stem cell niche should be used for cell culture. It might be able to help the isolated stem

cells maintain their myogenic capability before transplantation. The potential to alter the stem cell niche has attracted much recent attention from stem cell biologists (44; 45).

In the present study, we demonstrate that M-cadherin-mediated signaling suppresses GSK-3 β activation, promotes β -catenin/canonical Wnt signaling, and plays a positive role in maintaining the mitochondrial integrity in myoblasts during myogenic differentiation. Since both GSK-3 β and mitochondria play a crucial role in glucose metabolism, the regulation of M-cadherin on both could have profound effects on the glucose metabolism and insulin sensitivity of muscle tissues. Thus, it is possible that increased expression of M-cadherin may be a potential therapy to reduce the effects of metabolic syndrome or diabetes. Our speculation here is in concert with studies from various groups: Ectopic expression of M-cadherin was found in the liver of KK/Ta mouse, which is an established animal model for type 2 diabetes (46); E-cadherin may regulate insulin secretion by modulating the intercellular communication between β -cells within pancreatic islets (47; 48); In addition, activation of Wnt signaling promotes mitochondrial biogenesis and regulates insulin sensitivity in C2C12 myoblasts (49). Although therapeutic applications of the results of this study are likely several years away, the data we present in this study cast M-cadherin in a new role as a potential metabolism regulator, and therefore a potential therapy for addressing diabetes and/or the metabolic syndrome.

4.3. Future studies. Although we demonstrated that M-cadherin-mediated signaling plays a critical role in regulating myoblast fate and biological behavior in the

research that we completed in Chapter 2 and 3, there are still many questions that remain to be elucidated.

Firstly, we showed that M-cadherin promotes β -catenin/canonical Wnt signaling-mediated myogenic differentiation in a TCF/LEF-independent manner. Based on the studies from other groups, MRFs such as MyoD, as well as a MRF-regulatory protein I-mfa, could be the candidates that mediate the TCF/LEF-independent myogenic induction effect by M-cadherin/ β -catenin signaling. It has been shown that β -catenin interacts directly with MyoD and this interaction promotes MyoD transcription activity (50) and the binding of β -catenin to I-mfa relieves its inhibition to MRFs (51). To further identify the downstream target for M-cadherin/ β -catenin signaling, primary muscle progenitor cells could be isolated from hind limb muscles of wild-type mice. The change in gene expression level of MRFs and I-mfa could then be examined in cells treated with either M-cadherin RNAi or recombinant M-cadherin-Fc, to find out if the gene expression of any MRF and I-mfa are responsive to the manipulation of M-cadherin-mediated signaling. Additionally, the co-immunoprecipitation of β -catenin with MRFs and I-mfa could be performed to examine the change in interactions between β -catenin and MRFs and I-mfa in response to the manipulation of M-cadherin-mediated signaling. Furthermore, the gene expression of MRFs and I-mfa could be manipulated by overexpression or knockdown techniques to see which one could rescue the myogenic phenotype caused by M-cadherin RNAi. By doing so, the role of MRFs and I-mfa in mediating M-cadherin/ β -catenin's myogenesis-inducing effect will be clarified (Figure 24).

Secondly, one of the ultimate approaches is to establish a muscle-specific M-cadherin overexpression transgenic mouse model. The M-cadherin cDNA could be cloned into a transgenic vector plasmid driven by a muscle-specific muscle creatine kinase (MCK) promoter (52). The chimeric transgene should then be purified and used for generation of muscle-specific transgenic mice. Upon successful generation, the transgenic mice and the control ones with same genetic background should be used for following two sets of experiments. First set: cardiotoxin could be injected into the hind limb muscles of either the young or old, M-cadherin-overexpressing transgenic or wild-type (WT) mice, to cause muscle injury. One to two weeks after cardiotoxin injection, the injured muscles could then be dissected and markers for muscle regeneration and fibrosis could be analyzed and compared among young and old, M-cadherin-overexpressing and WT mice (Figure 25). In the second set of experiments, satellite cells could be isolated from the hind limb muscles of either M-cadherin-overexpressing or WT 2-week-old mice for in vitro culture. Myogenic differentiation could then be induced and markers for apoptosis, myogenesis, and fibrosis will be measured and compared between M-cadherin-overexpressing transgenic and WT cells (Figure 25). Through these proposed experiments, the findings from in vitro cell culture work that M-cadherin protects myoblasts against apoptosis and promotes myogenic differentiation will be verified at a gross muscle level in an animal system. These proposed studies could allow future students to clarify the role of M-cadherin in regulating adult muscle regeneration.

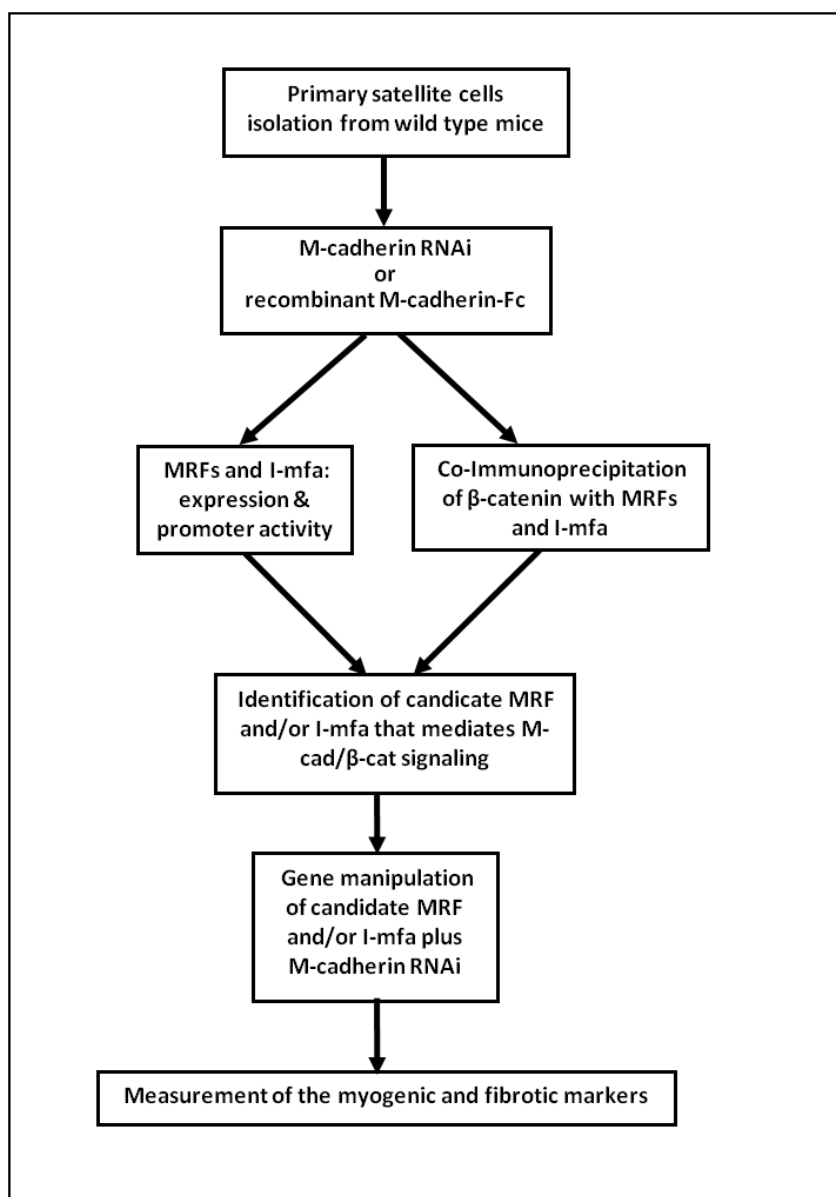


Figure 24. Outline of the strategies to clarify the role of MRFs and I-mfa in mediating M-cadherin/β-catenin's promoting effect on myogenic differentiation.

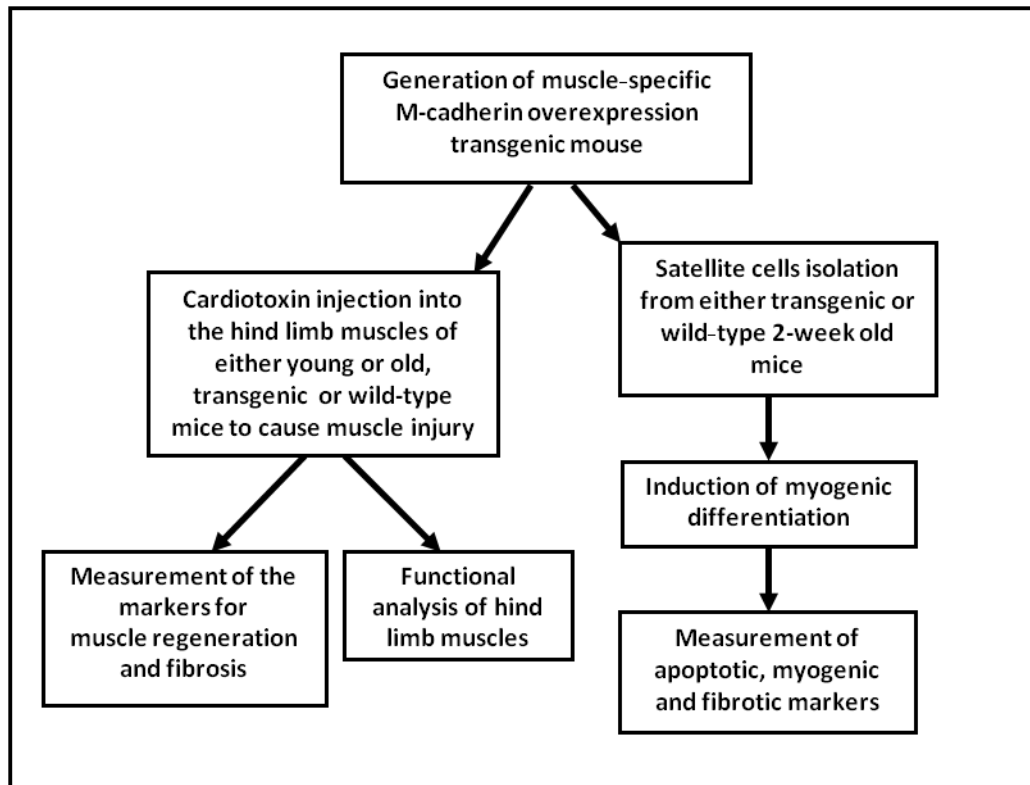


Figure 25. Outline of the strategies to examine the capability of muscle regeneration and fibrosis potential of M-cadherin (M-cad)-overexpression transgenic mouse in response to muscle injury, and the in vitro myogenic capability of satellite cells isolated from the transgenic mice.

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-

PUBLICATIONS

I. Skeletal muscle stem cell biology and muscle physiology:

1. **WANG Y**, Hao Y, Alway SE. Survivin Protects C2C12 Myoblasts against Oxidative Stress-induced Apoptosis. (in preparation)
2. **WANG Y**, Hao Y, Alway SE. M-cadherin-Mediated Signaling Modulates Phosphorylation of β -Catenin N-terminus and Promotes Myogenic Differentiation in a TCF/LEF-Independent Manner. (submitted to ***PLoS ONE***)
3. **WANG Y**, Hao Y, Alway SE. Suppression of GSK-3 β Activation by M-cadherin Protects Myoblasts against Mitochondrial-associated Apoptosis during Myogenic Differentiation. ***J Cell Sci*** (in press).
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II. Basic and clinical researches in skin diseases:

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ABSTRACTS PRESENTED

1. **WANG Y** and Alway SE. M-cadherin Signaling Maintains the Balance between Apoptosis and Myogenic Differentiation of Muscle Stem Cells by Suppressing GSK-

3 β activity. Poster presentation. Skeletal Muscle Satellite & Stem Cells 2010, Carefree, AZ.

2. Hao Y, Jackson JR, Scheller EY, **WANG Y**, Edens N, Pereira SL, and Alway SE. Effects of beta-hydroxy-beta-methylbutyrate on markers of muscle hypertrophy and apoptotic signaling during reloading in aged rats following disuse. *Med Sci Sports Exerc* 42 (5), Supplement S532:592. American College of Sports Medicine Annual Meeting, 2010, Baltimore MD.
3. **WANG Y** and Alway SE. M-cadherin Signaling Maintains Mitochondria Integrity of Muscle Stem Cells during Myogenic Differentiation via PI3K/Akt-1/GSK-3 β pathway. poster presentation. Making Muscle in the Embryo and Adult, a joint meeting of Frontiers in Myogenesis and Skeletal Muscle Satellite and Stem Cells, 2009, New York, NY.
4. **WANG Y** and Alway SE. M-cadherin Signaling Maintains Mitochondria Integrity of Muscle Stem Cells during Myogenic Differentiation via PI3K/Akt-1/GSK-3 β pathway. poster presentation. Experimental Biology 2009, New Orleans, LA.
5. **WANG Y** and Alway SE. M-Cadherin Protects C2C12 Myoblasts against Serum-Starvation-induced Apoptosis via PI3K/Akt-1/GSK-3 β Pathway. oral presentation, American College and Sport Medicine Annual Meeting 2008, Indianapolis, IN.
6. **WANG Y** and Alway SE. M-cadherin Mediates Myogenic Differentiation by Suppressing Id2 via β -catenin. poster presentation, Experimental Biology 2008, San Diego, CA.
7. **WANG Y** and Alway SE. N-cadherin Protects C2C12 Myoblasts from Serum-Starvation-induced Apoptosis. poster presentation, Experimental Biology 2007, Washington DC.
8. **WANG Y**, Li P, Chen HD, et al. Studies on the quantity of nerve fibers and their relationship with Langerhans cells in the lesional skin of psoriasis vulgaris. oral presentation, IX International Congress of Dermatology, 2004, Beijing.

INVITED PRESENTATIONS

1. Characterization of M-cadherin's role in regulating myoblasts apoptosis/survival and myogenic differentiation by interacting with key components of canonical Wnt signaling. **WANG Y**, NIEHS, NIH, Research Triangle Park, NC, August 25, 2010.
2. M-cadherin-mediated signaling regulates myoblasts biological behavior and fate specification by interacting with key components of canonical Wnt signaling. **WANG Y**, Cleveland Clinic Lerner Research Institute, Cleveland, OH, September 23, 2011

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